INFECTIOUS DIARRHOEA IN YOUNG ANIMALS

by

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Thesis submitted for the degree of

Doctor of Science

of the University of Edinburgh

1988

CONTENTS

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

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Degree D.Sc. *Date* JANUARY 1988

Title of Thesis INFECTIOUS DIARRHOEA IN YOUNG ANIMALS

No. of words in the main text of Thesis

The aetiology of infectious diarrhoea in young animals, particularly calves, was investigated, using techniques appropriate to the detection of viral, bacterial and protozoal pathogens. Rotavirus was established as of prime significance, and the classic 'white scour' syndrome in calves was usually caused by rotavirus with, on occasion, the simultaneous involvement of coronavirus or Cryptosporidium. Enterotoxigenic Escherichia coli (ETEC) infections were much less common, with under 6% of E. coli isolates possessing K99 fimbriae. Outbreaks of cryptosporidiosis were described for the first time from the U.K. The technique for the detection of the characteristic migration pattern of rotaviral double-stranded RNA segments in silver-stained polyacrylamide gels has proved of especial value in diagnostic and epidemiological investigations.

Infection of gnotobiotic lambs with lamb rotavirus produced dullness, inappetance, and diarrhoea, and provided a most useful model for pathogenesis studies. A rapid and extensive infection and defoliation of small intestinal epithelium leading to partial villus atrophy was followed within 2-3 days by a return to apparent morphologic normality. However, the underlying continuing dysfunction of an increased cell turnover rate was demonstrated by metaphase accumulation. Animals with acute enteritis were tolerant to levels of lactose normally found in milk, but their ability to digest and absorb increased oral doses of lactose was impaired. In calves, a concurrent rotavirus infection facilitated intestinal ETEC colonisation beyond the normal age of resistance.

Studies on passive immunisation in young lambs demonstrated that protection against rotavirus infection by antibody in the gut lumen was more effective than that provided by circulating antibody. The potential value of this technique was shown in experiments in lambs using rotavirus and immunoglobulin of human origin. Experimental adjuvanted vaccines of inactivated rotavirus given to ewes and cows in pregnancy significantly increased the titre of antibody of IgG1 isotype in colostrum and milk. Neonates ingesting these secretions were protected to various degrees against rotavirus infection and diarrhoea. The incorporation of commercially-produced K99 fimbriae from ETEC allowed the successful experimental testing and subsequent field trialling of a vaccine which substantially reduced rotavirus and ETEC diarrhoea problems in the progeny of vaccinated cows. Serological variation in rotavirus strains was of potential significance to successful vaccination: atypical rotaviruses with no serological relationship to 'conventional' rotaviruses were identified and characterised serologically and genomically, but occurred too infrequently in calves to present a major clinical problem. Distinct calf rotavirus serotypes that did not confer passive cross protection were identified. Cows produced a heterotypic immune response to all serotypes to which they had pre-existing antibody after vaccination with a single serotype. Passive immunisation may therefore largely overcome the practical problems posed by the existence of many rotavirus serotypes.

In the course of this work on neonatal diarrhoea, studies on diagnosis, epidemiology, pathogenesis and biochemistry of other enteropathogens, particularly astrovirus, Cryptosporidium, E. coli and Campylobacters were made. A method for exploiting the genetic control of susceptibility of piglets to adhesion with K88 fimbriae from ETEC was devised and tested.

INTRODUCTION AND ACKNOWLEDGEMENTS

It has been the candidate's great good fortune to have been caught up in the exciting and remarkable advances made in the field of neonatal diarrhoea of man and animals, during the period of research for this thesis from 1975-1987. It has been doubly fortunate to combine interest in this area with the privilege of working at the Moredun Research Institute. The work has been pursued with the beneficial, benign, and benevolent support of three successive Institute directors, Drs. John Stamp, Bill Martin and Ian Aitken. The Institute as a whole has an unusual sense of unity, so in a very real way my thanks for academic stimulation are due to all the scientific staff. The neonatal diarrhoea research programme has been a team effort involving colleagues in all departments of the Institute and from other establishments, and my debt to those whose names appear as co-authors is clear and gratefully acknowledged. However, it is appropriate to thank in particular Ken Angus, Eddie Gray and Iris Campbell for the significance and duration of their stimulation, encouragement and help.

SECTION 1

Aetiology of diarrhoea

While diarrhoea occurs commonly in animals of all ages it is apparent that clinical disease has a higher prevalence in the first few days and weeks of life. Among farm livestock, particularly severe problems are associated with intensive calf and piglet rearing, while diarrhoea in lambs is usually a lower morbidity problem.

The ubiquity of the bacterium *Escherichia coli*, and the failure to detect other significant bacteria or viruses by available techniques, led to the term colibacillosis becoming synonymous with diarrhoea. Studies in the 1960s by Williams Smith and colleagues began for the first time to differentiate strains of *E. coli* with diarrhoea-causing potential - first *in vivo*, then in ligated intestinal loops - and finally by demonstration of adhesins and toxins. This provided a considerable advance in understanding the aetiology of diarrhoea in piglets, but was less satisfactory for calves and lambs due to the relatively uncommon occurrence of these enterotoxigenic *E. coli* (ETEC) strains - indeed Williams Smith considered that physiological rather than infectious factors must be a primary cause (1).

A complementary advance was provided by Mebus at the University of Nebraska, who demonstrated that bacteria-free faecal filtrates from scouring calves caused diarrhoea on oral inoculation to germ-free calves. He then took the simple but revolutionary step of examining these faeces by electron microscopy (2). This led directly to the discovery of rotaviruses, a contribution of major importance to human and veterinary medicine, and indirectly by application of similar techniques to the discovery of several other pathogenic enteric viruses.

Taken together, the definition of virulence determinants in certain strains of E. coli and the discovery of rotaviruses, provided a major stimulus to research scientists interested in the aetiology of neonatal diarrhoea in man and animals. It was against this background that in 1975 the candidate commenced studies on neonatal diarrhoea. Demonstration of rotaviruses in lambs was made initially on morphological criteria by electron microscopy, supported by evidence of a serological relationship to calf rotavirus (Paper 1). The subject of enteritis in young lambs was reviewed (Paper 2).

The techniques which had been used to investigate diarrhoea in lambs were then developed and applied to the economically more significant problem of calf diarrhoea.

It was considered important to develop specific assays to detect the range of enteropathogens known or suspected from published work to be potentially important - rotavirus, coronavirus, ETEC, Salmonella sp, Campylobacter sp, and Cryptosporidium; while at the same time attempting to investigate outbreaks with a broad microbiological and histological approach to avoid preconceptions limiting the diagnostic range. This diagnostic interest resulted in the description for the first time in the UK of outbreaks of cryptosporidiosis in calves (Papers 3 and 4), which has given rise to a major research interest at Moredun. Conversely, the importance of ETEC was shown to be much less than previously perceived, with only 5.7% of 1529 E. coli isolates possessing K99 fimbriae (Paper 5). Isospora suis infection in scouring piglets was also described in the U.K. for the first time (Paper 6).

These studies culminated in a microbiological survey of calf diarrhoea carried out in Scotland and the north of England, at the same time as a similar agreed approach was undertaken in Wales and the south of England by colleagues at the AFRC Institute for Research on Animal Diseases, Compton, Berkshire (3). The classic syndrome of 'white scour' was shown to be caused by rotavirus, coronavirus, or Cryptosporidium, singly or in combination, with rotavirus predominating; ETEC caused a less common and clinically distinct acute watery diarrhoea in very young

calves; and no evidence was obtained that campylobacters were associated with diarrhoea (Paper 7).

This series of studies on the aetiology of diarrhoea in young livestock established a firm foundation of expertise for several derivative research areas, which will be discussed in the following sections.

The development of sensitive, accurate, and economical diagnostic techniques has been of obvious importance. The detection of the characteristic pattern of migration of rotavirus dsRNA genome segments by silver-staining polyacrylamide gels after electrophoresis (Paper 8) is now widely used in medical and veterinary diagnosis. The simplicity of the technique belies its great value in sensitive and accurate diagnosis, and in providing epidemiological information on strain variation.

Bovine coronavirus is less readily diagnosed than rotavirus and techniques used have often lacked specificity and sensitivity. The recent development of an immunogold-labelling electron microscopic technique provides a definitive diagnostic tool against which other techniques may be evaluated (Appendix paper A).

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SECTION 2

Rotavirus pathogenesis

The basic features of the pathogenesis of rotavirus infection in calves, with infection of villus epithelial cells leading to sloughing and partial villus atrophy, had been described (4). The facilities and expertise available at Moredun Research Institute offered the potential for further investigation of rotavirus pathogenesis.

With the existence of a complex normal and largely undefined abnormal intestinal microflora, the desirability of using germfree experimental animals to demonstrate rotavirus pathogenic potential was clear. In initial experiments to establish the model of infection, a bacteria-free filtrate of lamb faeces containing rotavirus produced virus multiplication, dullness, inappetance, and diarrhoea in day-old gnotobiotic lambs, but these features were all reduced in severity in 12-day old lambs (Paper 9). This rapid development of resistance with age observed to varying extents in many species has bedevilled experiments on active immunity to rotavirus.

A more detailed pathological study utilised samples collected from gnotobiotic lambs killed serially

throughout the infection and recovery periods (Paper 10). Tissues were examined by histology, immunofluorescence, and transmission electron microscopy. Epithelial cell infection and histological change were widespread throughout the intestine, from duodenum to colon, although maximal in jejunum and ileum during the incubation period and early clinical disease.

The pathophysiological mechanisms of acute rotaviral diarrhoea could reasonably be ascribed in general terms to disturbed digestion and absorption due to widespread enterocyte damage and loss. However, these lesions healed rapidly, while diarrhoea continued for several days, and increased enterocyte turnover rate was postulated in paper 10 as a mechanism for continuing gut malfunction. This was investigated further in studies on epithelial cell kinetics using a metaphase accumulation technique (Paper 11). Significant and prolonged increases in cell turnover were demonstrated, peaking 8 days after infection and continuing for at least 15 days. Thus, prolonged epithelial cell immaturity with consequent impaired function provided a possible explanation for the unthriftiness sometimes observed in calves after initial infection.

Brush border disaccharidases are depleted in the small intestine during rotaviral infection, but clinical

significance in the milk-fed neonate attaches only to lactase. A transient lactase deficiency was confirmed (Paper 12), but this was not reflected in intolerance to intake of physiological levels of lactose. This questions the rationale for the common practice of withholding milk during treatment of cases of calf diarrhoea.

As many enteric pathogens are endemic and conditions conducive to the spread of one enteropathogen also favour dissemination of others, it is not surprising that concurrent infections are common. Most interest has been taken in the possibility of interaction between rotavirus and ETEC, and experimental studies in gnotobiotic calves have variously shown a synergistic interaction, or merely an additive effect (reviewed, paper 13). It was felt that microbial interaction could more realistically be appraised against the background of a normal enteric flora, rather than in gnotobiotes. Thus experiments involving oral administration of rotavirus and ETEC separately and together were performed in 6-day old conventionally-reared calves (Paper 14). In spite of difficulties in microbiological control, the microbiological and pathological results showed that rotavirus infected the gut epithelium and produced diarrhoea, and that prior or simultaneous rotavirus

infection was probably necessary to enable ETEC colonisation in calves of that age.

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SECTION 3

Rotavirus immunology and vaccination

The candidate has pursued a particular interest in the immunology of rotavirus infections. One of the pioneering papers on calf rotavirus described the development of a live attenuated vaccine for young calves (5) which has achieved widespread use but little scientific acceptability. The most common postulate for the failure of this vaccine in the field is the neutralisation of vaccine virus by antibodies in normal bovine colostrum.

The candidate's studies have therefore concentrated on the principles and practice of passive immunisation.

The initial experimental model was once again the gnotobiotic lamb. In the young ungulate, born free of circulating immunoglobulins, there exists the unusual opportunity to separate the effects of circulating and local intestinal antibodies. Ingestion of colostrum in the neonate provides antibodies for absorption and hence systemic immunity, and a reservoir of local intestinal immunity. However in the young lamb ingesting post colostral milk, the absorbed circulating antibodies were not capable of protecting against rotavirus infection

once the antibodies in the intestine were removed. In contrast, the continued ingestion of small volumes of either colostrum or serum containing rotavirus antibodies could protect lambs through the continued presence of antibody in the intestine (Paper 15). This was of seminal importance for subsequent vaccine development, and was analogous to the concept of lactogenic immunity in transmissible gastroenteritis virus infection of pigs.

A further experiment in gnotobiotic lambs using human rotavirus and human immunoglobulins took this concept further by showing protection was mediated by IgG (Paper 16), thus confirming the specific nature of the protection. One of the most useful observations made in papers 15 and 16 and subsequently confirmed in other experiments, was that lambs passively protected against diarrhoea could nevertheless excrete rotavirus and become actively immune in their own right - so called "passive-active immunisation".

In Paper 17, the variables of antibody dose and titre were investigated. Intraperitoneal inoculation of serum also conferred protection, suggesting transfer of IgG1 across the gut wall.

These initial studies on rotaviral passive immunisation led to the attempted exploitation of the key

observation that specific antibody continually present in the gut lumen was protective against rotavirus diarrhoea. The first experiments in dam vaccination to study the antibody response in serum and milk were carried out in ewes. A simple vaccine of inactivated lamb rotavirus given intramuscularly to pregnant ewes produced a response characterised by a marked and persistent increase in serum neutralising titre and an increased titre of IgG antibody in colostrum and milk after lambing (Paper 18). The source of the milk antibodies was presumed to be the circulating IgG1 pool, with the implication that local stimulation of antibody-producing cells in the mammary gland was not occurring, in spite of the presumed natural intestinal priming with rotavirus.

These experiments in passive immunisation of lamb rotavirus infections have been reviewed in a paper presented at a Colloquium on Selected Diarrheal Diseases of the Young at the National Institutes of Health (Paper 19).

This initial and successful experiment in dam vaccination was extended and investigated in greater detail in cattle (Paper 20). The basic vaccine formulation used i.e. the UK strain of calf rotavirus grown in trypsin-treated rolled cell monolayers, inactivated with formaldehyde, and emulsified with a

Freund's incomplete-type adjuvant, was similar in this and subsequent experiments up to and including commercial vaccine formulation. Significant IgG1 antibody responses in serum and colostrum, and in milk for at least 30 days after calving, were demonstrated.

In the same experiment calves were challenged with rotavirus at 1 week of age and infection and disease were delayed but not prevented. These disappointing results were probably due to excessive challenge dose, but it is also possible that the presence in the calf-passaged challenge virus of 2 rotavirus serotypes (J.C. Bridger, personal communication) may have contributed.

Concern was felt about the protective capacity of antibody of IgG1 isotype, in view of results showing superior protection of piglets against transmissible gastroenteritis virus with antibody of IgA isotype (6). Again the ewe/lamb model was used, with post-colostral milk from vaccinated ewes at 5 and 12 days after parturition being selected to ensure a predominantly post-colostral IgG1 content (Paper 21). This milk fed as a dietary supplement completely protected gnotobiotic lambs against both rotavirus infection and diarrhoea, in contrast to a similar supplement of normal ewes' milk.

This experiment confirmed that ruminant IgG1 could protect the neonatal intestine against rotavirus.

It was considered that the result of further challenge experiments would depend on the stochiometric relationship between antibody titre and the dose of challenge virus. The most important and unknown variable was the size of the challenge dose in natural rotavirus outbreaks, the determination of which necessitated field trials. The first field experiment was performed with variation in some of the minor protocols, as it was carried out during an exchange visit to Australia e.g. the Northern Ireland rotavirus strain was used and 2 vaccinations were given. Continued feeding of colostrum as a dietary supplement for 14 days significantly decreased the severity of diarrhoea and increased liveweight gain in a natural outbreak of diarrhoea (Paper 22).

At this stage it was considered that a bivalent vaccine would be of greater value for field use, so a combined vaccine with rotavirus and K99 fimbriae from ETEC was developed with Wellcome Biotechnology. The rotavirus-neutralising antibody response to the combined vaccine was unimpaired, and the K99 antibody response was both significant and protective (Paper 23).

The ensuing field trials of the rotavirus/K99 vaccine under Animal Test Certificate illustrated the known difficulties of vaccine trials against infectious agents contributing to a syndrome of multiple aetiology, and of unpredictable occurrence (Paper 24). In particular, many farms with recurring annual scour problems experienced minimal disease after vaccination of half the cows, and cryptosporidiosis caused diarrhoea in several herds. However, in spite of suggestions elsewhere that such trials are not possible and that reliance has to be placed on historical controls, evidence of efficacy against both rotavirus infection and diarrhoea was obtained. No natural ETEC infection was encountered.

This vaccine is now experiencing commercial and field success as Rotavec K99 (Coopers Animal Health).

At the time these studies were initiated, the extent of serological variation among rotaviruses had not been investigated. After the description of viruses that on morphological and genomic criteria were rotaviruses, and yet which lacked any serological cross-reactivity with previously-described (group A) rotaviruses, it became important to investigate the occurrence of these 'atypical' rotaviruses. Three rotavirus groups were described, distinguished by serology and RNA electrophoretype, involving rotaviruses from man, calves,

piglets and lambs (Paper 25). However, these atypical rotaviruses are generally of low prevalence in disease, and their distinct terminal gene sequences makes reassortment with group A rotaviruses unlikely (7), so they are probably of little relevance to successful vaccination.

Of greater concern is the question of serotypes within group A rotaviruses, defined by cross-neutralisation tests, and determined by two outer capsid proteins (VP7 and VP3). Most field strains from calves in the UK were shown to share the same serotype, but the existence of at least 2 further distinct serotypes was confirmed (Papers 26 and 27). In addition, passive protection was shown to be serotype-specific, but the occurrence of a heterotypic immune response in vaccinated cows may be sufficient to ensure the success of monovalent rotavirus vaccination. Serotypic variation and heterotypic immunity are being investigated further.

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SECTION 4

Other infectious causes of neonatal diarrhoea

In the course of these studies on neonatal diarrhoea, several other known or potential enteropathogens were encountered, and studies have been made by the candidate on astrovirus, *Cryptosporidium*, *Escherichia coli*, and *Campylobacters*.

Astrovirus

The novel diagnostic technique of electron microscopic examination of faeces that became widely adopted for rotavirus diagnosis led also to the observation of other viruses and virus-like particles in faeces. One of the morphologically most distinct of these novel viruses has been widely and without dissension termed astrovirus since its initial description in 1975 (8) although no official approval exists for this terminology.

Astroviruses were detected in lamb faeces and transmitted in bacteria-free filtrates to germ-free lambs, confirming that astroviruses were indeed animal viruses (Paper 28). This initial study and a subsequent more extensive investigation (Paper 29) revealed the very mild

nature of the clinical disease produced experimentally, and the partial nature of the villus atrophy. The infected intestinal epithelium used as a source of antigen in immunofluorescent tests showed no cross-reaction with antisera to bovine and human astroviruses.

An ultrastructural study of lamb astrovirus in intestinal epithelium was made (Paper 30). The site of virus multiplication was confirmed as the mature enterocyte in the small intestine, with astrovirus entry through apical pits and tubules.

Biochemical studies were performed on extracts from infected intestinal epithelium due to the failure to cultivate lamb astrovirus in vitro (Paper 31). The genome of astrovirus was single-stranded RNA with similarities to picornaviruses and caliciviruses. However, the possession of 2 polypeptides each of about 33000 mol. wt. distinguished astrovirus from both these other groups.

A review paper on astroviruses was published in 1981 (Paper 32).

Cryptosporidium

Experimental cryptosporidiosis was studied in suckling mice and several variables relevant to the

infecting organism and the host were evaluated (Paper 33).

Cryptosporidium was not universally accepted as a primary intestinal pathogen, as transmission experiments were invariably accompanied by an extensive normal and, through lack of definition, potentially abnormal, intestinal microflora. For this reason, a strain of Cryptosporidium from calves was passaged through a series of 3 gnotobiotic lambs under intensive oral antibiotic treatment. The final faecal preparation, containing Cryptosporidium oocysts but no detectable bacterial or viral contaminants, produced a disease in gnotobiotic lambs similar to that previously described (Paper 34). Thus the primary enteropathogenicity of Cryptosporidium was confirmed. The opportunity was taken in this study also to describe the histological and ultrastructural changes in intestine, and the development of the parasitic lifecycle.

The subject of cryptosporidiosis was reviewed in an editorial for The Lancet in 1984 (Paper 35).

E. coli

It was necessary for the candidate to become involved to a small extent in bacterial research in order to study comprehensively the aetiology of calf diarrhoea.

An investigation into the occurrence of novel pathogenic mechanisms in E. coli identified several isolates from calves that produced a shiga-like toxin (Paper 36).

The genetic control of the receptor on piglet intestinal epithelium for adherence by E. coli with K88 fimbriae, and the relationship between adhesion and susceptibility to infection and diarrhoea (9), have not been exploited in any practical manner. Animals of nonadhesive phenotype from within the elite breeding nucleus of a large pig herd were identified by testing siblings and progeny at slaughter (Paper 37). This enabled resistant piglets to be produced from within the herd's existing breeding stock.

Campylobacter

Campylobacters isolated from young calves were investigated for pathogenic potential in gnotobiotic calves and lambs (Paper 38). Only subclinical disease characterised by alterations in faecal consistency and minor pathological changes was produced.

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A rotavirus in lambs with diarrhoea

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A reovirus-like agent was identified from an outbreak of enteritis in young lambs. From its morphology and immunological relationship with calf rotavirus, it was concluded that it was a rotavirus which infects lambs.

REOVIRUS-LIKE PARTICLES have been associated with acute gastroenteritis in human infants (Bishop *et al* 1973), and similar viruses cause diarrhoea in calves (Mebus *et al* 1969), pigs (Woode and Bridger 1975) and mice (Much and Zajac 1972). The name rotavirus has been suggested for this group of viruses (Flewett *et al* 1974), which are morphologically identical and which have been shown to be antigenically related (Woode and Bridger 1975; Kapikian *et al* 1974). A morphologically similar virus named the 'O agent' was isolated from mixed intestinal washings of cattle and sheep collected in an abattoir in South Africa, but the species from which the virus originated was not known, nor was it associated with disease (Els and Lecatsas 1972). This communication is the first report of a rotavirus in sheep.

Materials and methods

An outbreak of disease occurred in a group of 41 specific pathogen free lambs. There were six sudden deaths when the lambs were three days old, and post mortem examination showed congestion and subserosal haemorrhage of the ileum. All other lambs had soft yellowish faeces, and 11 more lambs died by the ninth day. All survivors were treated with streptomycin daily from the fourth to ninth day, and no deaths occurred after the ninth day.

Faeces were collected from the diarrhoeic lambs, and intestinal contents from the dead lambs. Specimens were prepared for electron microscopy (EM), by making either a 10–20 per cent suspension of faeces in distilled water, or by clarification of this suspension by centrifugation at 10,000 *g* for 30 min, followed by centrifugation of the supernatant fluid at 150,000 *g* for 45 min, and resuspending the pellet in a few drops of distilled water. In each case, a drop of the suspension was transferred to a carbon-coated grid, and stained with 1 per cent potassium phosphotungstic acid (pH 7·4).

For immuno-electron microscopy, lamb virus preparations or calf faeces known to contain rotavirus were suspended in distilled water, then briefly ultrasonicated to disperse any aggregates of virus particles. Antiserum, prepared in gnotobiotic calves, to calf rotavirus was kindly provided by Dr G. N. Woode, Institute for Research on Animal Diseases, Compton, and lamb serum was obtained from surviving lambs one week after recovery. Equal volumes of virus preparation and serum dilutions (1/10–1/100) were mixed and left at room temperature for 60 min, then stained and examined by EM.

Primary calf kidney cell cultures with flying coverslips were inoculated with lamb virus filtrates, as described by Woode *et al* (1974). Coverslips were removed and stained

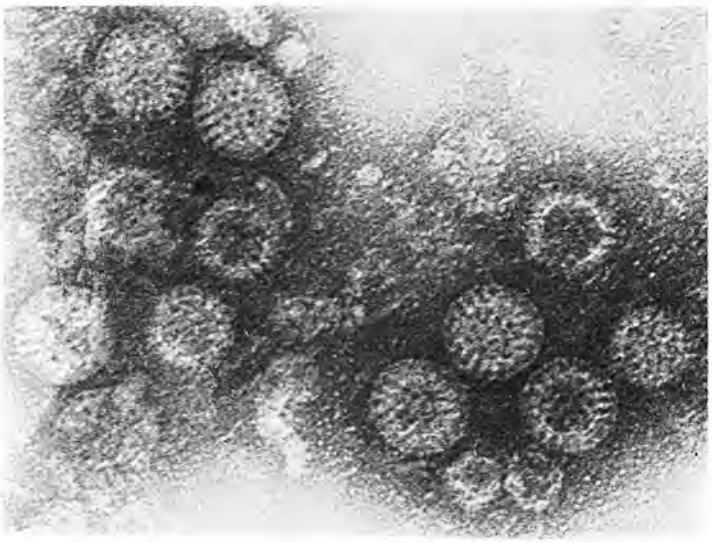

FIG 1: Rotavirus in lamb faeces × 200,000

by indirect immunofluorescence, using calf antiserum to calf rotavirus, and fluorescein-conjugated rabbit anti-bovine globulin (Nordic).

Results

Numerous virus particles were detected in the gut contents of two of the three dead lambs examined by EM, but not in the third. The virus was identical in morphology with those described as rotaviruses (Flewett *et al* 1974). Two sizes of particle were observed, those of 68–70 nm diameter with an outer coat, and others of 58–60 nm diameter in which no outer coat was visible (Fig 1). Some particles had an electron dense hexagonal core of approximately 40 nm.

Faeces were collected from 11 of the lambs when they were eight and nine days old. Virus particles similar to those described above were observed in 10 of the samples, and in seven of these they were in sufficient numbers to be visible without concentration. Faeces were collected from the same 11 lambs when they were 15–16 days old, but only small numbers of virus particles were found in concentrated preparations from two lambs.

Virus particles from the lambs were seen on EM to be agglutinated by calf rotavirus antiserum, but not by fetal calf serum. Strands of globulin were seen linking the virions, particularly those without the outer capsid layer. Similarly, sera from two convalescent lambs caused agglutination of both lamb and calf virus particles.

Calf kidney cell cultures inoculated with lamb virus showed specific intracytoplasmic fluorescence with calf rotavirus antiserum, but not with fetal calf serum.

Klebsiella aerogenes or *Proteus vulgaris* were isolated from faeces of four of the six lambs which died and all the 35 survivors when they were examined on the fourth day.

Discussion

The morphology of this lamb virus, and its r reaction by immunoelectron microscopy and imm fluorescence with calf rotavirus, indicate that it rotavirus. Its relation to the outbreak of enteritis desc is more difficult to assess. The fact that it was found of 11 sick lambs, but in only two of the same 11 l after recovery, strongly suggests that it was involv causing the disease. However, as potentially patho bacteria were also isolated from most lambs, the ent may have been due to a combined action of the rota and bacteria.

Studies are continuing, to evaluate the significan this rotavirus in diarrhoea of lambs.

Received for publication June 24,
Accepted July 21,

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8 ENTERITIS IN YOUNG LAMBS

D. R. SNODGRASS AND K. W. ANGUS

It has been estimated that between 2 and 4 million lambs die each year in the UK. Most of these losses are not due to infectious diseases of the young lamb but to abortions and stillbirths on the one hand and starvation or chilling of the lamb on the other. However, of the infectious conditions affecting the lamb, diseases of the alimentary tract are the most important.[1] Enteric diseases most commonly manifest as diarrhoea and can result in significant mortality or economic loss from reduced condition, drugs, and labour. Many flocks, even those on extensive husbandry systems throughout the rest of the year are kept intensively at lambing time. This produces ideal conditions for a build-up of infectious neonatal

disease: close confinement, gradual increase of contamination and a continual through-put of susceptible young animals to amplify infectious agents. The fact that lambing takes place in winter or early spring means that adverse environmental factors such as cold, wet, or windy weather, are prevalent and these conditions are often associated with diarrhoea outbreaks.

Causes

No comprehensive surveys have been published to enable assessment of the relative importance of different infectious agents. However, studies have been made of individual micro-organisms, and these are described separately.

LAMB DYSENTERY

Caused by *Cl. perfringens (welchii)* type B (see chapter on Clostridial Diseases)

ESCHERICHIA COLI

E. coli is a normal inhabitant of the bowel of sheep. By the second day of life, the healthy lamb excretes 10^9–10^{10} *E. coli*/g of faeces, and this count decreases gradually with age until a plateau of about 10^6–10^7 *E. coli*/g is reached in the adult sheep. Only a minority of strains of *E. coli* are capable of causing diarrhoea and knowledge of the virulence factors involved is well documented. Early experiments to assess the enteropathogenicity of *E. coli* were performed by either oral inoculation of neonatal lambs, or by inoculation of ligated intestinal loops. Isolates capable of causing diarrhoea in the experimental lambs also caused fluid accumulation in isolated intestinal loops. Techniques to identify virulence factors have advanced, and it is recognized that *E. coli* capable of causing diarrhoea possess an antigenic pilus (known as K99 and common to calf and lamb isolates) which enables them to adhere to intestinal epithelium and produce an enterotoxin, which in calf and lamb strains is usually a heat-stable, low molecular weight, non-antigenic toxin (ST). Isolates dilating ligated gut loops, or possessing K99 and ST, are considered enterotoxigenic *E. coli* (ETEC).[2]

ETEC have been isolated from 20–43 per cent of scouring lambs in surveys in the UK and the USA. Affected lambs are usually less than one week old, and exhibit fluid diarrhoea, dehydration, and weakness. Mortality rates up to 75 per cent have been reported.

SALMONELLAE

Salmonellosis is a sporadic cause of enteritis and loss in young lambs. Individual outbreaks are often very severe with fever, abortions, diarrhoea and high mortality in sheep in affected flocks. The major serotypes implicated are *S. dublin* and *S. typhimurium*, though more exotic serotypes e.g. *S. orienburg*, have been isolated in some incidents. Evidence from recent surveys suggests that in the UK *S. dublin* is the predominant serotype in ovine enteric salmonellosis.

The primary source of infection where *S. typhimurium* is involved is often difficult to identify, since this serotype can cause disease in a wide range of host species. With *S. dublin*, however, direct or indirect contact with infected cattle must be considered as a possible initiating cause.

The first indication of disease is frequently sudden death with no premonitory clinical illness, though dead lambs may display yellow staining of the perineum.[3] Sick animals are dull, febrile and refuse to suck; a greenish-yellow diarrhoea which may be blood-stained is commonly present. Older lambs are often thirsty, and may be found dead beside a source of water. The course of clinical illness is brief and death supervenes within 24 hours.

Necropsy findings in young lambs are often inconsistent. Abomasal and small intestinal contents are usually watery, and the macroscopic appearance of the intestinal mucosa varies from apprently normal to obviously inflamed over considerable lengths. The abomasal mucosa is often severely inflamed, with focal hyperaemia or haemorrhage in the plicae. Signs of dehydration may be present.[3] Mesenteric lymph nodes may be enlarged and oedematous, and salmonellae can normally be cultured from these, the small intestine, spleen and liver.

CAMPYLOBACTERS

All strains of *Campylobacter* associated with acute enteritis belong to a group with thermophilic characteristics, currently classified as *C. fetus* subs. *jejuni/coli*. Although numerous strains of the organism have been found in sheep faeces, there is little evidence that they present a hazard to young lambs although regional ileitis resulting in unthriftiness has been

reported in older lambs.[4] Attempts to induce clinical disease in lambs by oral transmission of *Campylobacter* strains from scouring calves have been unsuccessful, although infection was established. It is possible that persistent infection acquired in the neonatal period is responsible for a small proportion of unthrifty lambs at around weaning age.

ROTAVIRUS

Viral enteritis attracted much interest in the 1970s, and viruses are now regarded as significant causes of diarrhoea. Rotavirus in particular has been shown to occur globally in man and his domesticated mammals and birds.[5] Rotaviruses from different species are serologically related, but are usually distinguishable by genetic and antigenic analysis. Transmission of rotavirus from one host species to another has been frequently accomplished experimentally, but the existence of natural zoonotic transmission has not been confirmed. In particular, reports of diarrhoea in farm children at lambing time have not so far been adequately investigated.

The existence of a rotavirus from lambs has been confirmed and in the only published survey rotavirus infection was detected in 25 per cent of scouring lambs.[6] Antibody surveys suggest that rotavirus is endemic in the sheep population, as has been found in man, cattle and pigs.

Lamb rotavirus has not so far been adapted to grow in cell culture. However, experimental studies using gnotobiotic or colostrum-deprived lambs have enabled pathogenesis to be studied. As in other species, lamb rotavirus infects and destroys the mature absorptive villus epithelial cell of the small intestine, leading to villus atrophy and a malabsorptive diarrhoea.

OTHER VIRUSES

Conventional virological techniques based on tissue culture result in the isolation of enteroviruses, adenoviruses, and reoviruses from lamb faeces. Little significance is normally attached to these isolates, but reovirus type 1, ovine adenovirus type 1, and bovine adenovirus type 2 have all been associated with respiratory and enteric disease in lambs in large intensive farms in Hungary.[7, 8]

Studies based on electron microscopic examination of lamb faeces have been minor compared with the extensive investigations into diarrhoea in calves and piglets, with the result that comparatively little information is available. Astrovirus has been described from an outbreak of lamb diarrhoea, and has been shown experimentally to induce a mild diarrhoea in gnotobiotic lambs. However, there is no evidence that astrovirus is an important pathogen. Coronavirus-like viruses have also been described from lambs, but their morphology is distinct from true coronaviruses infecting calves and piglets and their significance is unknown.

CRYPTOSPORIDIUM

Endogenous stages of *Cryptosporidium*, a small protozoon of the enteric coccidia group, adhere to the microvillous borders of intestinal epithelial cells. The parasite differs from other coccidia in that it has a short (2–4 days) life-cycle, and apparently lacks host specificity.[9] Transmission is via infected faeces. Under experimental conditions, isolates from scouring calves, lambs and red deer infected gnotobiotic lambs, causing clinical diarrhoea with widespread cryptosporidial infection and severe damage in the intestine, particularly the ileum. An outbreak of cryptosporidiosis in artificially-reared lambs has been described, with severe diarrhoea and deaths[10] and more recent evidence suggests that serious outbreaks can occur in naturally-reared lambs at about 7–10 days old. However, the prevalence is unknown. Affected lambs quickly become dull and anorectic. They develop a 'tucked-up' appearance, and become stiff and reluctant to keep up with their dams. There is no febrile reaction. The length of clinical illness varies but is usually at least 7 days, before gradual recovery commences. Some lambs die after 2–3 days illness, others appear to recover, then relapse. Surviving lambs remain unthrifty for several weeks.

Necropsy findings are often vague: the carcass is usually thin and may be dehydrated. The intestines are flaccid, but the mucosal surface may appear normal, though in some instances there may be congestion in the distal small bowel. The caecum is often distended with khaki-coloured liquid contents, while the spiral colon is often empty. Histological examination shows widespread infection of the villous epithelium, particularly in the distal jejunum and ileum, with widespread atrophy and fusion of villi. In prolonged infections, the caecum and colon and sometimes the rectum, may be infected with the parasite, resulting in a severe typhlitis and colitis.

Diagnosis

To establish the aetiology of an outbreak of diarrhoea in lambs requires laboratory investigation, and the possibility of lamb dysentery should not be ignored. Salmonellosis typically presents as a wider syndrome than neonatal diarrhoea alone, and if salmonellosis is suspected, conventional bacteriological techniques to isolate *Salmonellae* should be employed.

ETEC may be isolated from faeces on blood agar and McConkey agar. No correlation exists between haemolysin production and enteropathogenicity of calf and lamb strains of *E.coli*. K99 expression can be prevented by excess polysaccharide production, so passage of *E.coli* on minimal medium such as Minca + 1% Isovitalex prior to testing for K99 by slide agglutination is preferred. Heat stable toxin is normally assayed by inoculating cell-free culture fluids to infant mice. Ideally, to confirm a diagnosis of ETEC infection, the bacteria should be shown to be present at abnormally high titres in the small intestine i.e. $> 10^8$/g. To demonstrate this, an acutely-ill untreated lamb should be sacrificed, *E. coli* counts made, and the presence of K99-producing *E. coli* adhering to intestinal epithelium demonstrated by immunofluorescence of cryostat sections of small intestine.

Rotavirus infections are more readily diagnosed, and the technique most widely used is ELISA performed directly on faeces samples. Sections of intestine obtained as above can also be stained for rotavirus immunofluorescence. In the absence of specific immunological reagents, examination by electron microscopy is satisfactory and offers the added advantage of a catch-all technique for several other enteric viruses. Briefly, an approximate 20 per cent suspension of faeces in saline is made and the coarse debris allowed to settle. A drop of supernatant fluid is transferred to an electron microscope grid, stained with a negative stain to delineate virus particles, and examined in an electron microscope at a magnification of 40 000–50 000. Alternatively, the faecal suspension may be clarified by lowspeed centrifugation and the supernatant fluid centrifuged at high speed to pellet viruses. The pellet is then resuspended in a small volume and examined. However, no consistent increase in sensitivity has been shown with this method. Rotaviruses have a characteristic morphology, and can often readily be recognized in large numbers from specimens prepared as described (Fig. 8.1).

To detect cryptosporidia in faeces smears on glass

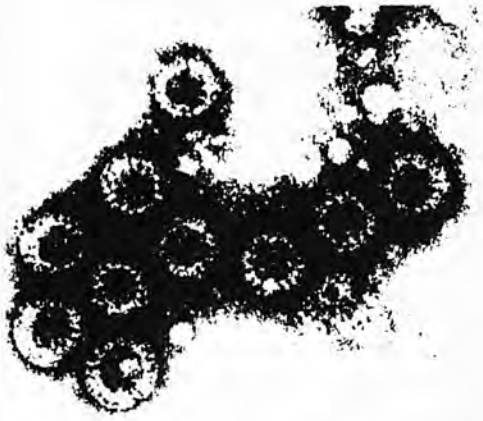

Fig. 8.1 Rotavirus particles in faeces. The characteristic arrangements of the outer capsomeres giving the impression of spokes radiating from a wheel hub led to the adoption of the name rotavirus. (x 102 000)

Fig. 8.2 Cryptosporidia (arrowed) in Giemsa-stained faecal smear (x 4250)

slides are air-dried, fixed in methanol, and stained by Giemsa's method. Examination under x100 objective lens is necessary to detect the cryptosporidia, which are circular structures stained blue with no obvious capsule 3–4 μm in diameter, with often several eccentrically-placed pink-staining granules, and characteristically an unstained halo around the organism (Fig. 8.2). Faeces containing cryptosporidia are also infectious for suckling mice. Cryptosporidia can be readily demonstrated in histological sections of both small and large intestine from samples fixed shortly after death (Fig. 8.3). Up to 24 hours after death, cryptosporidia may be observed in mucosal scrapings.

Fig. 8.3 Cryptosporidia attached to enterocytes in ileum of a naturally-infected calf. (x 1000)

Treatment and control

It is important to confirm if lamb dysentery is involved, so that appropriate prophylactic measures can be carried out, (see Chapter 7). Immunization against salmonellosis is unsatisfactory, although killed vaccines are useful on premises where a particular serotype has already been diagnosed. In the absence of lamb dysentery and salmonellosis, disease produced by the other micro-organisms involved can probably not be distinguished clinically. However, as there is no specific vaccine or therapy for any of these micro-organisms, control depends on non-specific measures. The lambing area should immediately be moved to a clean site to attempt to break the build-up of infection. If lambing takes place indoors it is frequently difficult to change lambing areas, but in that case young lambs should be moved outside as quickly as weather permits. Extra attention should be paid to the nutrition of the ewe, to ensure adequate colostral production, and to the care of young lambs to ensure adequate colostral intake.

No controlled trials of the use of antibiotics in lambs have been made, but by analogy with other species their use is probably of little value. Effective fluid replacement therapy and warming of sick lambs are the most appropriate remedies. Although sulphonamides have been occasionally reported effective in bovine cryptosporidiosis, this has not been confirmed.

It is possible that vaccines being developed for use in calf diarrhoea could eventually be applied in sheep. These vaccines are intended for use in pregnant cows, to stimulate production of antibody in colostrum and milk to rotavirus and ETEC. Experiments in ewes and lambs have shown the validity of a similar approach, and an effective K99-based ETEC vaccine for ewes is feasible.[11] Whether a calf rotavirus vaccine given to ewes passively protects lambs against lamb rotavirus is not known, although antibodies to calf rotavirus in cows' milk have been shown to protect piglets against pig rotavirus infections. A separate lamb rotavirus vaccine is not at present feasible as lamb rotavirus has not been adapted to grow in cell culture.

'WATERY MOUTH'

Watery mouth is a colloquial name for a clinical entity responsible for sporadic but sometimes quite heavy losses in young lambs in the immediate post-

natal period. Recent reports based on close observation of flocks where watery mouth is a recurrent annual problem[12, 13, 14] give a perspective on the clinical features and circumstances surrounding occurrences.

Lambs 12 h to 7 days old may be affected, but the highest incidence is in the 12–48 h age group. The condition is commonest in multiple births, but can occur in singles. Frequency of occurrence is greater in lambs born towards the end of the lambing period than in early lambs. The clinical expression of the condition varies from farm to farm and from year to year on the same farm, but may be so severe as almost to reach epidemic proportions.

Affected lambs do not suck and quickly become dull, lethargic and comatose. The muzzle and lower jaw are often wet with saliva or regurgitated stomach contents, and the mouth feels cold. The abdomen is often distended: lambs have a bloated appearance. Diarrhoea is absent; rather, lambs appear constipated, with no passage of the meconium. On handling the abdomen, a marked gurgling sound due to accumulation of gas in the intestines may be heard, known locally as 'rattle belly'. Death usually takes place within 12–24 h.

At necropsy features consistently found include retained meconium and the presence of excessive amounts of thin clear mucin (fetal mucin) in the abomasum. If colostrum is present, this is unclotted. In slightly older lambs, the abomasum may be distended with milk. *E. coli* is usually isolated from the gut and other organs (septicaemic colibacillosis).

Treatment is aimed at overcoming intestinal stasis by removing the meconium using enemas and laxatives, and controlling infection with oral and parenteral antibiotics. In mild cases, laxatives alone may be sufficient to effect a cure, but in severe outbreaks oral antibiotics may have to be given to lambs at birth, and possibly repeated later, to prevent further losses. *E. coli* antiserum may be of some value in controlling losses.

Analysis of possible predisposing causes indicates that these are multifactorial. Undoubtedly, inadequate colostral intake is an important contributory factor. As this is related to diminished sucking drive, such contributing factors as hypothermia, hypoglycaemia, or prenatal infections, e.g. border disease, toxoplasmosis, or enzootic abortion have been suggested. Irrespective of cause, adequate colostral intake must be ensured, if these lambs are to survive.

Management factors are probably of equal importance on some farms. Penning of the lambs with their mothers for up to 12 h is an important predisposing factor, particularly when it seems likely that build-up of pathogenic enterobacteria is occurring as lambing proceeds. Prolonged penning should only be considered if the weather is particularly inclement. In the most adverse circumstances, the lambing-pens may have to be abandoned for the season, and disinfected during the summer. There is also evidence that early castration or docking with rubber rings predisposes to watery mouth.

Preventive measures thus depend on individual circumstances. Attention to the management factors discussed above almost certainly helps to reduce the incidence in most circumstances. Chemoprophylaxis with oral antibiotics, though doubtless satisfactory in the short term, can only lead to further development of transmissible drug resistance in strains of enterobacteria, and is fundamentally undesirable. Vaccination of the ewes with a combined *Pasteurella haemolytica/E. coli* vaccine 4 weeks before lambing has been used to control the condition.

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REPRINTED FROM THE VETERINARY RECORD, MAY 31, 1980

Cryptosporidia associated with rotavirus and an Escherichia coli in an outbreak of calf scour

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Veterinary Record (1980) **106.** 458-459

CRYPTOSPORIDIA are coccidia which parasitise the microvilli of intestinal epithelial cells. They have been found in outbreaks of calf scour in Canada (Morin and others 1976) and in the USA (Moon and others 1978, Pohlenz and others 1978) and were demonstrated in a colostrum-deprived experimental calf in the United Kingdom (Pearson and Logan 1978). This paper records for the first time in the UK the occurrence of cryptosporidia in an outbreak of calf scour.

The incident involved a herd of 240 blue-grey cows and heifers, all in calf to Aberdeen Angus bulls, on an upland farm in Lanarkshire, Scotland. Calving began in mid-July with cows and heifers kept separately in groups of 20 to 25 in fields of 5 to 8 hectares. No supplementary feeding was made available, nor was any attempt made to group pregnant animals according to gestational age. Consequently by mid-

FIG 1: Stunted villi in calf ileum, partly lined by cuboidal cells. Numerous cryptosporidia are just visible in the brush borders of the lateral villus epithelial cells at this magnification. Giemsa × 77

FIG 2: Higher magnification of calf ileum, showing cryptosporidia in the brush borders of enterocytes. Giemsa × 793 oil immersion

FIG 3: Cryptosporidium oocyst (arrowed) in faeces smear. Giemsa differentiated in alcoholic colophonium resin × 825 oil immersion. (Oocysts are approximately 4 μm in diameter, and are identifiable by the presence of four to six bright red granules in a pale blue matrix, when stained by this method)

August, when a scour problem in the calves became apparent, calves were being born into an existing calf population, some of which were already scouring.

Severe scour problems were encountered in calves born to heifers. These developed a profuse watery green diarrhoea starting five to seven days after birth. Initially, affected calves were bright, alert and consequently difficult to catch for treatment. However, after 24 to 48 hours they became extremely dehydrated, inappetant and dejected. The problem was further complicated by the very haphazard treatment methods employed by the farmer, different antibiotics being used at successive treatments and at varying dose levels.

Faeces samples were obtained from 13 untreated calves on the day of onset of diarrhoea, and from five unaffected calves. Rotavirus was detected in faeces from six of the scouring calves by electron microscopy (Snodgrass and others 1976) and enzyme immunoassay (ELISA) (Ellens and de Leeuw 1977) but not in any of the unaffected calves. *Escherichia coli* with demonstrable K99 antigen (Moon and others 1976) were cultured from two scouring calves and one unaffected calf. Sera from a sample group of calves contained adequate immunoglobulin (zinc sulphate turbidity range 28 to 54 units) and total protein (range 55·3 to 84·7g per litre) levels. Examination of sera from 12 periparturient cows and four calves revealed marginal serum copper status (range 40 to 76 μg per ml) in the herd.

One, four-day-old scouring calf was brought to the laboratory and biopsy specimens of small and large intestine, obtained under anaesthesia before euthanasia, were processed for light and electron microscopy. Changes were confined to the ileum, where partial atrophy of villi was widespread and many atrophic villi were lined by cuboidal epithelial cells (Fig 1). Numerous organisms resembling cryptosporidia were visible in the brush borders of enterocytes (Fig 2). Electron microscopy confirmed that these were the trophozoite, gametocyte and oocyst forms of the parasite, all partially embedded in the microvillus border of the cells. A second calf killed subsequently also had lesions associated with cryptosporidia in its ileum.

Examination of faecal smears stained by Giemsa's method revealed the presence of typical cryptosporidia oocysts (Pohlenz and others 1978) in a further two calves (Fig 3).

The next six calves to develop scour were treated with sulphadimidine at 5 g daily for three days. Early results were encouraging, in that faeces returned to normal consistency within 24 hours of initiating treatment. Within seven days, however, the treated calves started to scour again and the severe outbreak of diarrhoea continued in calves subsequently born, with little response to therapy. A total of 30 calves died in the outbreak. This emphasises the intractable nature of calf scour in herds such as this one, where at least three enteropathogenic organisms were present concurrently.

In this outbreak, there was direct demonstration of cryptosporidia in four calves, by either histological examination or examination of faecal smears. Since then, cryptosporidia have been detected by us in an outbreak of scour in another herd of suckled beef calves, in this latter case without detection of any other enteropathogen.

There are no data on the significance of cryptosporidia in calf scour in the UK. However, this parasite is known to occur relatively commonly in calves in North America, where it is considered to be an enteric pathogen (Moon and others 1978, Pohlenz and others 1978). Thus, yet another microorganism has to be considered in investigations into the aetiology of the calf scour complex.

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Printed in Great Britain by R. W. Simpson and Co. Ltd., 70 Sheen Road, Richmond, Surrey.

REPRINTED FROM THE VETERINARY RECORD, DECEMBER 20, 27, 1980

An outbreak of calf diarrhoea attributed to cryptosporidial infection

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Veterinary Record (1980) **107.** 579-580

COCCIDIAN parasites of the genus *Cryptosporidium* have been isolated from several hosts including mammals, birds and reptiles (Levine 1973) and probably have a world wide distribution. Cryptosporidia are extracellular organisms infecting the lower small intestine (Vetterling and others 1971, Barker and Carbonell 1974, Pohlenz and others 1978). Although there occurrence was reported by Tyzzer in 1907, only recently has their association with diarrhoea in calves (Meuten and others 1974, Pohlenz and others 1978, Snodgrass and others 1980a) and lambs (Berg and others 1978) been described. The significance of calf cryptosporidiosis has been difficult to assess in the cases reported (Pohlenz and others 1978, Snodgrass and others 1980a) because of the presence of other enteropathogens. A natural outbreak of calf diarrhoea in which cryptosporidia, the only enteropathogens detected, were probably the primary cause is reported here.

The outbreak occurred in a housed beef herd of 41 blue-grey sucking calves, born over a period of eight weeks. The herd, which had previous history of severe neonatal diarrhoea, was involved in a vaccination experiment. Half the cows (21) were vaccinated before calving with experimental calf rotavirus vaccine described by Snodgrass and others (1980b). Faeces were collected from each of the 41 calves on the sixth and 14th day after birth. Twenty-eight days after the first calving, when diarrhoea first started, faeces were also collected from scouring calves at the onset of the clinical illness. A total of 101 faecal samples was screened (62 normal, 39 diarrhoeic) for bovine rotavirus and coronavirus by enzyme-linked immunosorbent assay (ELISA) tests (Ellens and de Leeuw 1977), for other enteric viruses by electron microscopy (Snodgrass and others 1976) and for K99 possessing *Escherichia coli* (Moon and others 1976). Oocysts of cryptosporidium were observed in faecal smears stained with Giemsa (Pohlenz and others 1978).

Table 1 summarises data collected from the affected herd. Fig 1b shows the pattern of the outbreak in terms of the number of scouring calves on days after the first calving. The toal number of calves present on certain days is indicated. The distribution of scouring calves follows a near normal curve including a smaller pattern on the right (at 60 days) which represents a relapse in eight of the 35 scouring calves. Only six calves escaped diarrhoea. Fig 1a shows the number and distribution of faecal samples collected on the first day of diarrhoea from each of the 31 scouring calves tested and the proportion in which oocysts could be detected. In the 62 normal faecal samples neither oocysts, enteric viruses nor enterotoxigenix *E coli* were detected.

Generally, the calves experienced a mild to moderate degree of illness with occassional signs of anorexia and depression among the younger animals. Affected calves were treated daily with kaolin and pectin (Kaogel; Park Davis) and a solution of electrolytes, glycine and dextrose (Ionaid; Syntex Pharmaceuticals); kaolin and pectin and a solution of electrolytes and dextrose (Electrosol; Willington Medicals); kaolin and pectin and saline; or a sulphadiazine, sulphamerazine and sulphapyridine formulation (Trinamide; May & Baker), depending on age and severity of diarrhoea.

Positive diagnosis of cryptosporidiosis in calves has in the past been confirmed by the demonstration of parasites attached to the brush borders of the enterocytes. Since none of the calves died and no histopathological material was available we resorted to experimental inoculation of a calf which had been previously exposed to the most commonly encountered enteropathogens to eliminate the risk of natural infections during the experiment. A 10 ml suspension (10 per cent v/v) prepared from faeces containing oocyst was fed to a seven-day old colostrum-fed calf which had fully recovered from a previous dual infection with bovine rotavirus and enterotoxigenic *E coli* (Tzipori and Campbell unpublished data).

Nine days after inoculation the calf developed moderate diarrhoea with coincident shedding of oocysts in the faeces, at which time it was killed. Examination of haematoxylin and eosin stained gut sections revealed nemerous cryptosporidium attached to the brush borders of the intestinal enterocytes. The faeces and gut contents were screened for other enteropathogens by methods described above and by immunofluorecent staining. No other agent was detected in this calf throughout the incubation period or at post mortem examination.

TABLE 1: Summary of data collected from a suckler beef herd with suspected cryptosporidiosis

Number of calves in the herd	41	
Number of faecal samples examined	101	
Number of calves with diarrhoea	35	(85%)
Number of calves with diarrhoea tested	31	
Number of calves shedding oocysts	18	(60%)
Number of calves with recurrence of diarrhoea	8	(23%)
Number of calves with recurrence and shedding oocysts	4	
Number of normal faeces containing oocysts	0	
Youngest calf with diarrhoea (days)	5	
Youngest calf shedding oocysts (days)	6	
Oldest calf with diarrhoea (days)	39	
Oldest calf shedding oocysts (days)	26	
Mean age of onset of diarrhoea (days)	15·3	(±1·15)
Mean interval between two episodes of diarrhoea	7·1	(±1·51)
Mean duration of first episode (days)	7·7	(±0·73)
Mean duration of second episode (days)	4·4	(±0·60)
Age when recurrence began (days)	16·5	(±1·81)
Longest duration of diarrhoea (days)	16	
Correlation between age and duration of diarrhoea	Not significant	
Correlation between age and recurrence of diarrhoea	Not significant	

(± Standard error)

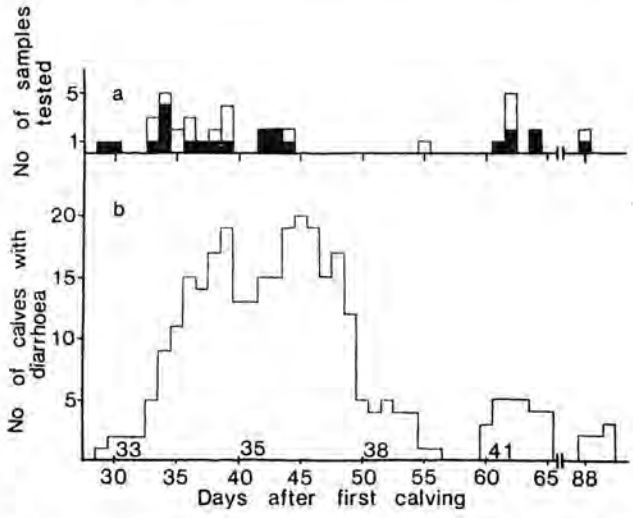

FIG 1: (a) Number and distribution of faecal samples collected on the first day of scouring from 31 calves (plus eight relapses) and the proportion which contained cryptosporidial oocysts (solid columns); (b) number of scouring calves for each day following first calving (numbers below the horizontal axis) and the number of calves present on certain days (numbers above the horizontal axis)

In this outbreak of calf diarrhoea cryptosporidium was the only demonstrable cause. The disease occurred in claves aged one week and older probably because of the relatively longer incubation period of the cryptosporidium compared to other reported enteropathogens affecting very young animals. The duration of the disease was also longer and calves usually scoured intermittently for at least two weeks. In a number of instances calves experienced a second episode of the disease one to three weeks later. Recurrence of diarrhoea following treatment has also been observed in scouring lambs with suspected cryptosporidiosis (Berg and others 1978). Regular treatment of the infected calves with antimicrobial agents may have modified the severity of the disease and could have reduced the number of oocysts shed.

It seems that cryptosporidium acting alone in this outbreak produced only mild to moderate diarrhoea without mortality. The significance of cryptosporidiosis in terms of its contribution to the enteric syndrome in young animals and its prevalence remains to be determined but from the observations reported must be considered in outbreaks of diarrhoea in calves.

Acknowledgements.—The authors thank the Institute for Research on Animal Diseases, Compton, Newbury, for screening faeces for bovine coronavirus and the staff of Glensaugh Research Station, namely M. Begg, D. Harrison and L. Fairlie, for observations, collection of material and recording of data.

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Printed in Great Britain by R. W. Simpson and Co. Ltd., 70 Sheen Road, Richmond, Surrey.

Papers and Articles

Prevalence of enterotoxigenic *Escherichia coli* in calves in Scotland and northern England

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Veterinary Record (1983) **113**, 208–212

Eighty-eight of 1529 (5·7 per cent) *Escherichia coli* isolates from diarrhoeic and clinically normal calves in Scotland and northern England were found to possess the K99 pilus antigen (K99$^+$). There was complete correlation between possession of K99 antigen, heat stable enterotoxin production and ability to dilate intestinal loops. The diagnosis of calf enterotoxigenic *E coli* infections may therefore be based on the detection of K99 antigen alone. Enterotoxigenic *E coli* was isolated from 23 of 306 (7·5 per cent) diarrhoeic calves from eight of 70 (11·4 per cent) farms and was not isolated from clinically normal calves. Infected calves were between one and three days old. A survey by an enzyme-linked immunosorbent assay found 3·0 per cent and 3·9 per cent of sera from calves and cows respectively to contain antibodies to K99 antigen. The prevalence of other enteropathogenic organisms in calf faeces is also discussed.

ESCHERICHIA coli has been shown to be a normal inhabitant of the gastrointestinal tract of animals and man (Smith and Crabb 1961, Smith 1962, 1965). Strains of *E coli* within the intestinal lumen are multiple and are continuously varying (A. H. Linton, personal communication). Thus the frequent assertion that *E coli* causes neonatal diarrhoea is difficult to confirm.

Early surveys of calf mortality incriminated *E coli* as a major cause of death due to 'white scours' (Jensen 1893, Jordan 1933, Lovell and Hughes 1935, Withers 1952) although the *E coli* strains isolated were not tested for their ability to cause experimental diarrhoea in calves. Later studies by Smith and Halls (1967a) showed that certain strains of *E coli* isolated from calves and piglets caused dilation of ligated gut loops and this effect correlated with the ability of the strain to cause diarrhoea. These enteropathogenic strains were shown to produce enterotoxins (Smith and Halls 1967b, Gyles and Barnum 1969) and hence were referred to specifically as enterotoxigenic *E coli* (ETEC).

The gut loop test revolutionised the search for ETEC in calf diarrhoea outbreaks, and in the past 15 years surveys have been conducted in North America and Japan (Acres and others 1975, Myers 1975, Moon and others 1976, Morin and others 1976, Sivaswamy and Gyles 1976, Isaacson and others 1978, Moon and others 1978, Nakazawa and others 1981), ETEC being isolated from as many as 30 per cent of diarrhoeic calves examined.

Most calf ETEC, apart from producing a heat stable enterotoxin active in the infant mouse assay, were shown to possess a gut adhesive (pilus-like) antigen, K99 (Orskov and others 1975, Burrows and others 1976) which was able to facilitate the adherence of ETEC to the small intestine of infected calves (Moon and others 1978). Occasionally, though, K99-positive calf *E coli* have been shown to produce a heat labile enterotoxin (Moon and others 1976, Isaacson and others 1978) and in one instance a K99-negative calf *E coli* isolate was shown to produce a Vero cell cytotoxin (Kashiwazaki and others 1980).

No surveys to date have established the prevalence of ETEC in calves in Britain. Data are presented in this paper on the prevalence and enteropathogenic characteristics of ETEC in the faeces of diarrhoeic and clinically normal calves from farms in Scotland and northern England. Also included are findings from a serological survey of K99 antibodies in the sera of cows and calves, and relevant information on the prevalence of other enteropathogenic organisms in the faeces of calves.

Materials and methods

Material for this survey was collected from 362 home bred calves (276 suckler and 86 bucket reared dairy calves) on 70 farms in Scotland and the north of England from October 1979 to April 1982. Faecal samples were taken from calves (up to 21 days of age) which had recently developed diarrhoea and before treatment had been initiated. Between two and 22 calves were sampled on each farm and on occasion faeces were collected from clinically normal calves. Faeces were collected and transported to the laboratory in sterile bottles. Five to 10 ml of faeces were obtained which enabled several examinations to be performed on one sample. Samples were cultured for *E coli* on the day of sampling or on the day of arrival if sent by post.

E coli *strains*

The following strains were used as controls in diagnostic tests: O101:K$^-$:K99 (B41), K12:K99 and K12 (supplied by Dr J. Morris, Weybridge, England), O148:K?:H28 (EC1) (supplied by Dr B. Rowe, Colindale, England) O9:K30:K99 (B44) and O26:K?:H? (H19) (supplied by Dr H. W. Smith, Houghton, England).

Isolation and identification of K99$^+$ E coli

Fresh faecal swabs were plated on 5 per cent sheep blood agar and MacConkey agar. After overnight incubation at 37°C, five representative lactose-fermenting colonies were selected and subcultured on minca-Isovitalex agar (Guinee and others 1977). After overnight incubation at 37°C the growth from each colony was tested for K99 antigen by slide agglutination using rabbit anti-K12:K99 serum absorbed with strain K12.

Three isolates from each calf were stored on Dorset egg medium slopes at 4°C for further tests. Representative lactose-fermenting colonies were confirmed as *E coli* using either the Sensititre (Seward Laboratories) of Micro-ID (General Diagnostics) tests.

Heat stable toxin test

Heat stable toxin was assayed in sucking mice by a

From September 14, veterinary medicines will come under the Medicines (Veterinary Drugs) (Prescription Only) Order 1983 (SI 1983 No 1213, £5.55). Human medicines will be dealt with under the Medicines (Products other than Veterinary Drugs) (Prescription Only) Order 1983 (SI 1983 No 1212, £6).

The change is an administrative one and does not affect the way medicines are prescribed or dispensed.

BEVA

Firing major topic at equine congress

TENDON injury and repair will be the subject of a major scientific session to be held at the British Equine Veterinary Association congress in York on September 6.

Professor Ian Silver of Bristol University presenting the controversial report for the Horserace Betting Levy Board on the use of firing and tendon splitting in the treatment of tendon injury, will open the symposium. Five other speakers will cover aspects of this important equine problem.

Besides opening the associations 22nd congress on September 5, HRH The Princess Anne Mrs Mark Phillips will be giving a paper on training event horses. Joining Her Royal Highness in a session devoted to training will be speakers on racing, fitness, training and nutrition of equines for different purposes.

Orthopaedics, law and insurance and radiography will be the subject of other main symposia during the congress.

Veterinary ethology

Animal behaviour examined

WAYS in which physiology modifies behaviour in animals and the effects of social contact were described by French, British, Dutch and Belgian workers at the summer meeting of the Society of Veterinary Ethology. It was held at the University of Tours and the INRA Institute of Physiology and Reproduction, Nouzilly, France in July.

The first theme, physiology and behaviour, attracted several interesting papers. C. Schaeffer and his colleagues reported work which suggested that the adrenal cortex was capable of producing a pheromonal substance which stimulates feminine behaviour in the male rat when ovarian hormones are missing.

C. Guyomarc'h described how she and her co-workers had investigated the sensibility of sexual development in female Japanese quail to stimulation by the male song. F. O. Odberg of Ghent's paper concerned stereotyped behaviour in voles and neurochemical correlation. A study of the interbreed differences in adaptive abilities in three breeds of Chinese pigs by M. Bluthe of Bordeaux and her colleagues had suggested that female pigs were generally more adaptive than males.

D. Pissonnier of Nouzilly, describing work done with colleagues, said that early recognition of the lamb by its dam depended on olfaction. This recognition became established within two hours of parturition during which time the dam formed a maternal bond selective to her own lamb and excluding all others. Their work suggested that bonding was at least partly dependent upon noradrenergic influences in the olfactory bulb.

Martin and Cognie, also of Nouzilly, said that seasonally anoestrous ewes of many breeds would ovulate in response to the introduction of rams. Working with Romanov ewes, their results had indicated that the effect of the ram might be more efficient at certain times of day, namely 20.00.

B. Cougouille-Gaffreteau had treated mares with androgen injections which caused them to rise in the hierarchic order and adopt the main characteristics of male sexual behaviour. Two months after the treatment ceased the mares had recovered their female hormone balance but interestingly, had kept the higher social rank they had acquired.

Mothering

After studying calves of two breeds, Friesian and a hardy French breed, Salers, D. le Neindre said that Salers needed to be mothered at birth but that Friesian calves were very active and adaptable and did not seem so responsive to their environment.

R. F. Parrott and B. A. Baldwin, who had been working with sheep at Babraham, showed that dihydrotestosterone had little, if any, central action in oestrogen treated wethers. Peripherally it enhanced growth and sensitivity of androgen sensitive tissues and permitted the full expression of behaviour induced centrally by dihydrotestosterone or oestrogen. Local stimulation of the preoptic region of the brain of male sheep with testosterone had induced sexual and agressive behaviour.

The second day's theme was social behaviour. M. Barton of Reading suggested that by allowing teat fed calves to feed simultaneously in close proximity, the effect of social facilitation was enhanced, increasing the intake of the less motivated calves and thus producing more even growth rates with restricted feeding.

Effect of light

G. van Putten of the Netherlands had kept pigs at three levels of light: dark, semi dark (1 lux) and brilliantly lit (25 lux). There were some statistical differences between pigs in the completely dark pens and the other two types of light. In the dark there was more recumbency, less social behaviour, less exploratory behaviour and more tail biting. These pigs also had more joint deformities and leg malfunctions.

On the final day, M. Meunier and J. M. Faure of Nouzilly, who had been working on the influence of feeder design on the feeding and social behaviour of laying hens, said that when only limited feeding space was allowed the main welfare problem was probably not increased competition but decreased synchronisation of the group.

M. Kiley-Worthington of Sussex University, speaking on the affect of different environments on the social interaction of horses, suggested that the most important contributory factors in the development of behavioural problems were a lack of roughage and lack of social contact.

The final paper, given by W. T. Jackson of Lewes, was on criminal cases relating to cruelty to animals. He discussed the importance of case law in England and other countries.

News in Brief

Parvovirus increase? Reports have been received from veterinary surgeons in several areas, including Kent, South London and Yorkshire, that considerably more cases of parvovirus than normal are being seen. The incidence is said to be particularly high in unvaccinated pups.

Charter veterinarians to be commemorated The family home of Thomas Mayer and his son, Thomas Walton Mayer, in Newcastle-under-Lyme is to be renamed 'Mayer House' by Mr D. L. Haxby, President of the RCVS, on Sunday, October 2 at a joint meeting of the Veterinary History Society and the Staffordshire Historic Buildings Trust. The two Mayers, who were both veterinary practitioners in Newcastle-under-Lyme, played an integral part in the events that led to the granting of the Charter of the Royal College of Veterinary Surgeons in 1844.

Disease outbreaks The following outbreaks of notifiable diseases occurred during the week ending August 7: *Aujeszky's disease*.– Crowan, Cornwall; Sahem Toney, Norfolk (two outbreaks); Goosargh, Lancashire; Hackford, Norfolk; Thurton, Norfolk; Fressingfield, Suffolk; Ashbocking, Suffolk; Wortwell, Norfolk; Rossendale, Lancashire; Queen Hulton, Greater Manchester; Swainsthorpe, Norfolk; Llanfetchel, Gwynedd; Brockdish, Norfolk. *Paramyxovirus of pigeons*.– Southampton, Hampshire; Carmamman, Dyfed.

Products and Services

Immobilon and Revivon transferred to C-Vet

C-VET Ltd acquired the veterinary business and goodwill of the products Immobilon and Revivon from Reckitt & Colman plc, on August 24. Immobilon and Revivon (large and small animal formulations) are used to induce reversible neuroleptanalgesia before minor operations.

Peter Simm, managing director of C-Vet, comments that during the transfer of the product licences, revised data sheets have been prepared with a number of changes made in the recommendations for use, particularly with regard to operator safety.

New appointments at Glaxo

MR Mike Parrott has been appointed marketing director of Glaxovet. He succeeds Mr Peter Hanbury, who retired in June. Mr Derek Allison has been appointed sales manager of Glaxovet.

Evans Animal Health, the company's subsidiary dealing with poultry, has appointed Mr Rob Garnett as general manager and Mr Philip Box as international veterinary adviser. Dr Ian Fleming has been appointed research manager for Glaxo Animal Health and Mr Ray Harding has been appointed manager of the new clinical trials unit.

modification of the method described by Dean and others (1972). *E coli* isolates were grown overnight in 10 ml of casamino acids-yeast extract broth (Evans and others 1973). After centrifugation of the broth at 1600 *g* for 20 minutes at 4°C the supernatant was membrane filtered (Millipore, 0·45 μm average pore diameter) and stored at −20°C.

Each supernatant when thawed and coloured with Evans blue dye (0·1 per cent w/v final concentration) was inoculated into three one- to four-day-old Schneider Swiss white mice. The inoculum was given in 100 μl volumes intra-oesophageally using a 26 gauge needle tipped with plastic tubing. After inoculation mice were kept at 15 to 18°C for four hours, then killed with chloroform. The intestines from duodenum to rectum were removed and weighed together for each group of three mice. The ratio of gut weight: remaining bodyweight was calculated as reported by Dean and others (1972); ratios less than 0·07 were considered negative, 0·07 to 0·08 questionably positive, and greater than 0·08 positive for the presence of heat stable toxin. Positive results were repeated for confirmation. The negative control contained casamino acids-yeast extract broth only and the positive control a supernate from the growth of strain B41.

Heat labile toxin test

Heat labile toxin was assayed on Y1 mouse adrenal tumour cells and the test was modified from the technique described by Donta and Smith (1974). Bacteria-free culture supernatants were prepared as decribed in the stable toxin test, but were tested on the day of preparation or after storage at -70°C for no longer than seven days. The negative control contained casamino acids-yeast extract broth only and the positive control a supernatant from the growth of strain EC1.

To confirm labile toxin identification, a neutralisation assay was used in which an equal volume of culture supernatant was incubated at 37°C with rabbit anticholera toxin (supplied by Dr S. Van Heynigen, Edinburgh, Scotland) for one hour before assay.

Vero cytotoxin test

Vero cytotoxin was assayed on Vero cells using a method modified from that of Konowalchuk and others (1977). Bacteria-free culture supernatants were prepared as described for the stable toxin test and were tested on the day of preparation. Casamino acids-yeast extract broth was used as a negative control and supernatant from the growth of strain H19 was used as positive control.

Calf ligated loop test

E coli isolates were inoculated into calf ligated loops as described by Myers and others (1975). *E coli* isolates were grown overnight in 10 ml trypticase soy broth at 37°C. The bacterial growth was centrifuged at 1600 *g* for 20 minutes at 4°C and the pellet resuspended in 2 ml fresh trypticase soy broth to contain approximately 10^{10} colony forming units per ml.

Jersey or Ayrshire calves five to 10 days old were used with 40 to 50 loops being made in the mid-gut of each calf. Loops were inoculated with 2 ml of bacterial suspension with two loops in each set of 10 containing control strains B44 (ETEC) and strain K12 (non-ETEC). Two loops were used per isolate, and after 18 to 24 hours a ratio of fluid accumulation: segment length of equal to or greater than 1·0 ml/cm was considered a positive response (Myers and others 1975).

Anaesthesia was induced and maintained with halothane and nitrous oxide, and subsequent sedation and analgesia was provided by xylazine (Rompun, 2 per cent solution; Bayer UK) and pethidine (50 mg/ml; Evans Medical) at intervals when necessary.

Calf brush border adhesion test

Brush borders were prepared from the small intestinal epithelium of a nine-day-old Ayrshire calf using the method of Sellwood and others (1975). The adhesion of *E coli* isolates to these brush borders was carried out using the method described by Sellwood and others (1975) with strain K12:K99 as positive control and strain K12 as negative control.

Serotyping

Representative K99$^+$ *E coli* isolates from each farm were sent to Drs I. and F. Orskov (Statens Seruminstitut, Copenhagen, Denmark) for O, K and H antigen typing.

Antibiotic sensitivity test

Twenty-seven K99$^+$ and 33 K99$^-$ *E coli* isolates were tested for their in vitro sensitivities to seven antimicrobial agents using Mastring-S discs (Mast Laboratories). The antibiotics were streptomycin (10 μg), gentamicin (10 μg), ampicillin (10 μg), polymyxin B (100 units), chlortetracycline (25 μg), chloramphenicol (25 μg) and neomycin (30 μg). Isolates were grown for three hours at 37°C in trypticase soy broth then inoculated to provide confluent growth on dextrose sensitivity test agar using a swab. The Mastring-S discs were applied to the media and the plates thereafter incubated at 37°C for 18 hours.

Antibiotic sensitivities were assessed from the diameter of the zone of inhibition of growth around the discs with reference to data presented in the Diamed Diagnostic manual (1981). Isolates with reduced or no zone of inhibition were considered to be resistant in the test, although it should be recognised that reduced zones of inhibition may be an influence of inoculum concentration and that the antibiotic may still be active at therapeutic levels.

Examination for other enteropathogens

Faeces were screened for enteric viruses by electron microscopy, rotavirus and coronavirus by enzyme-linked immunosorbent assay (ELISA) (Fahey and others 1981), coronavirus by haemadsorption-elution-haemagglutination assay (van Balken and others 1979), *Cryptosporidium* species in Giemsa-stained faecal smears (Sherwood and others 1982) and *Salmonella* species by overnight enrichment in sodium selenite broth.

Sampling of sera and testing for K99 antibodies

Sera from 304 calves aged one to six months which had been submitted to the virology section of the Moredun

TABLE 1: Summary of the characteristics of *E coli* isolated from diarrhoeic and clinically normal calves

Test	Number of isolates tested	Number isolates positive/number tested Isolates from diarrhoeic calves	Isolates from clinically normal calves
K99	1529	88/1273	0/256
STa	685*	61/605	0/80
LT	743*	0/633	0/110
VT	28*	1/28	NT
Gut loops	35*	16/35	NT

K99 K99 antigen detected by slide agglutination
STa Heat stable enterotoxin detected by infant mouse assay
LT Labile toxin detected by Y1 mouse adrenal tumour cell assay
VT Vero cytotoxic factor
NT No isolates tested
* Isolates selected from the 1529 tested for K99 antigen

Research Institute during 1981 for diagnostic purposes unconnected with this investigation were examined for K99 antibodies. Sera were also taken from 179 cows from 13 farms (11 to 30 samples per herd) in which calf diarrhoea had been considered a problem.

Sera were assayed for K99 antibodies by ELISA (Snodgrass and others 1982a).

Results

A summary of the characteristics of 1529 *E coli* isolates from diarrhoeic and clinically normal calves is given in Table 1.

K99 antigen tests

Eighty-eight of the 1529 isolates tested for K99 antigen (5·7 per cent) were positive ($K99^+$). All $K99^+$ *E coli* were isolated from calves with diarrhoea (23 of 306 diarrhoeic calves, 7·5 per cent). No $K99^+$ *E coli* were isolated from 56 clinically normal calves. From 21 of the calves excreting $K99^+$ *E coli*, all the isolates tested were $K99^+$ (Table 2).

Heat stable toxin tests

Six hundred and eighty-five isolates were tested for stable toxin activity, and 61 were found to be positive (STa^+). All STa^+ *E coli* were isolated from 23 of 279 diarrhoeic calves. Fig 1 shows two populations of *E coli* isolates as determined by the infant mouse assay, STa^+ *E coli* (mean gut: bodyweight ratio of 0·101) and STa^- *E coli* (mean gut: bodyweight ratio of 0·057).

All STa^+ *E coli* were $K99^+$, while no STa activity was detected in $K99^-$ *E coli*. Henceforth *E coli* which are STa^+ and $K99^+$ will be referred to as ETEC.

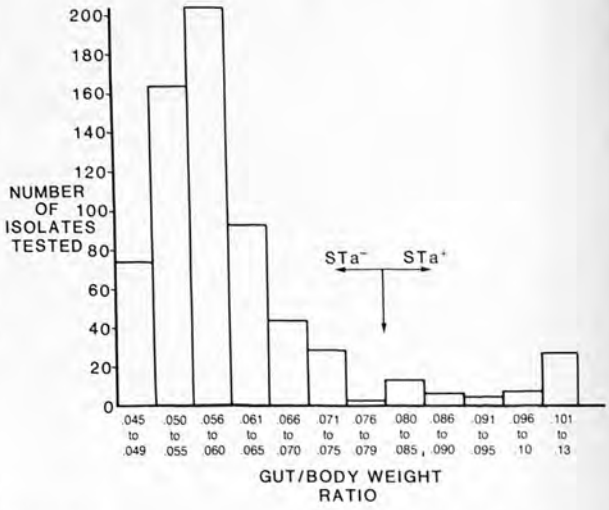

FIG 1: Infant mouse assay for heat stable toxin (STa). Distribution of the gut:bodyweight ratios of mice inoculated with bacteria-free culture supernatant from the growth of 685 calf *E coli* isolates

Heat labile toxin tests

Eight of 743 isolates caused cell rounding in the Y1 mouse adrenal tumour cell assay. These isolates comprised six of 110 strains tested from clinically normal calves and two of 633 isolates tested from diarrhoeic calves and originated from five farms. The active factor was heat labile (100°C for 10 minutes) but cell rounding was not neutralised by specific rabbit anticholera toxin. Immunologically therefore, the

TABLE 2: Characteristics of $K99^+$ *E coli* isolated from diarrhoeic calf faeces

Farm	Calf number*	Number positive/number tested: K99	STa	LT	VT	Hly	Serotype	Antibiotics to which strains were resistant†
1	E346	5/5	3/3	0/3	NT	5/5	O8:K85:H27	S
	E349	5/5	3/3	NT	NT	5/5	NT	NT
	E350	5/5	3/3	NT	NT	5/5	NT	NT
	E351	5/5	3/3	0/3	NT	5/5	O8:K85:H27	NT
	E352	3/5	1/1	NT	NT	3/5	NT	NT
	E354	2/5	1/1	NT	NT	2/5	O8:K85:H27	NT
	E355	5/5	3/3	0/3	NT	5/5	NT	P, S
2	9051/1	3/3	3/3	0/3	NT	3/3	NT	S, A, Ct
	9051/2	3/3	3/3	0/3	0/1	3/3	NT	S, A, Ct
	9051/3	3/3	3/3	0/3	NT	3/3	NT	NT
	9076/1	3/3	3/3	0/3	NT	3/3	O8:K85:H27	S, Ct
	9076/2	3/3	3/3	0/3	NT	3/3	NT	NT
3	B/H	5/5	3/3	0/3	0/1	5/5	O8:K85:H27	S, A, Ct, C
4	B2625/3	5/5	3/3	0/3	0/3	5/5	O8:K⁺:H?	S, A
	B2625/4	5/5	3/3	0/3	0/3	5/5	NT	S, N
	B2625/5	5/5	3/3	0/3	1/3	5/5	NT	S, N
	B2625/6	5/5	3/3	0/3	0/3	5/5	NT	S, N
5	B2649/1	3/3	3/3	0/3	0/3	3/3	NT	S
	B2649/3	3/3	3/3	0/3	0/3	3/3	O8:K85:H⁻	S
6	D650/1	5/5	3/3	0/3	0/3	5/5	O8:K85:H?	S
7	D679/1	5/5	3/3	0/3	0/3	0/5	O101:K28:H⁻	S, A, Ct
8	C56/1	1/1	1/1	0/1	0/1	0/1	O141:K85ab:H?	S, A
	C56/2	1/1	1/1	0/1	0/1	0/1	O141:K85ab:H?	S, A

STa Stable toxin active in infant mice
LT Labile toxin
VT Vero cytotoxin
Hly Haemolysis produced on sheep blood agar
NT Not tested
H? Only weakly motile
* All calves from suckler herds
† S = streptomycin, A = ampicillin, P = polymyxin B, C = chloramphenicol, Ct = chlortetracycline, N = neomycin

effect induced by these isolates was not due to classical labile toxin. The nature of the factor that caused this rounding of Y1 adrenal cells was not identified. None of the ETEC isolates produced labile toxin.

Vero cytotoxin tests

Only one of 28 ETEC isolates tested was positive in this test. This isolate was from farm 4 (Table 2).

Calf loop tests

Sixteen ETEC isolates evoked positive reactions (1 to 8 ml/cm) in at least one loop, but 19 randomly selected non-ETEC isolates were consistently negative. Six of the eight isolates that caused rounding of Y1 adrenal cells were examined and found to be negative.

Brush border adhesion tests

Twenty-one randomly selected ETEC isolates adhered to calf intestinal brush borders in vitro, but no adherence was observed with any of 36 randomly selected non-ETEC isolates.

Serotyping analysis

ETEC were isolated from calves on eight of 70 farms sampled (11·4 per cent). Eleven K99$^+$ ETEC strains from these eight farms were examined (Table 2) and five different serotypes were found: O8:K85:H27 (five isolates from farms 1 to 3); O8:K$^+$:H? (one isolate from farm 4); O8:K85:H$^-$ (two isolates from farms 5 and 6); O101:K28:H$^-$ (one isolate from farm 7); and O141:K85ab:H? (two isolates from farm 8).

Haemolysin production

There was a good but not absolute correlation between enterotoxigenicity and haemolysin production (Table 3). In the serotyped isolates, haemolytic activity was associated with the O8 serogroup only.

Antibiotic resistance of ETEC and non-ETEC

A higher proportion of non-ETEC than of the ETEC isolates was resistant to five of the antibiotics tested, but the reverse was the case for streptomycin and neomycin (Table 4).

Age distribution of ETEC infections

All ETEC infections occurred in calves under three days old, diarrhoea commonly starting within 24 hours of birth. On six farms (farms 3 to 8, Table 2) ETEC were the only enteropathogens isolated. On two farms (farms 1 and 2) there were concurrent ETEC and rotavirus infections in some of the calves examined. The dually infected calves became diarrhoeic between 24 and 72 hours old.

TABLE 3. Correlation of enterotoxigenicity with haemolysin production

	Total number of isolates	ETEC isolates	Non-ETEC isolates
Hly$^+$	86	81*	5
Hly$^-$	1443	7	1436

Hly$^+$ Haemolytic
Hly$^-$ Non-haemolytic
* Significant correlation by χ^2 ($P < 0{\cdot}01$) of enterotoxigenicity with Hly$^+$

TABLE 4: Antibiotic resistance of ETEC and non-ETEC isolated from the faeces of calves

Antibiotic	Percentage of strains demonstrating resistance: Non-ETEC (33 isolates)	ETEC (27 isolates)
Streptomycin	69·7	100 **
Gentamicin	0	0
Ampicillin	42·4	30
Polymyxin B	3·0	0
Chlortetracycline	75·5	22·2***
Chloramphenicol	24·2	3·7*
Neomycin	26·7	37·5

* $P = 0{\cdot}07$; ** $P = 0{\cdot}005$; *** $P = 0{\cdot}001$ by χ^2 test

Other enteropathogens detected

Rotavirus was detected in 90 diarrhoeic calves (29·4 per cent) and six clinically normal calves (10·7 per cent, $P < 0{\cdot}01$). Seventeen diarrhoeic calves (5·6 per cent) and four clinically normal calves (7·1 per cent) were excreting coronavirus (P not significant). *Cryptosporidium* species were present in the faeces of 43 diarrhoeic calves (14·1 per cent) and four clinically normal calves (7·1 per cent, P not significant). No *Salmonella* species were detected.

Twenty-one diarrhoeic calves (6·9 per cent) had mixed infections comprising ETEC and rotavirus (three calves), coronavirus and rotavirus (five calves); *Cryptosporidium* species and rotavirus (12 calves) and *Cryptosporidium* species, rotavirus and coronavirus (one calf). Including ETEC isolations at least one enteropathogen was detected in 148 of 306 diarrhoeic calves (48·3 per cent) and 14 of 56 clinically normal calves (25·0 per cent).

Serological survey for K99 antibiodies by ELISA

Seven of 179 cow sera tested (3·9 per cent) contained K99 antibodies. Four of these cows came from one farm and the remainder from three separate farms.

Nine of 304 calf sera tested (3·0 per cent) contained K99 antibiodies. Four of these calves came from one farm and the remainder from five separate farms.

Discussion

ETEC were isolated from the faeces of 7·5 per cent of diarrhoeic calves on 11·4 per cent of the farms studied (all suckler herds). There was 100 per cent correlation between K99 antigen production, elaboration of stable toxin and dilation of calf ligated gut loops by ETEC. Previous studies have shown good but not complete correlation between K99 antigen and stable toxin, with some stable toxin-producing isolates being shown to be K99$^-$ (Guinee and others 1976, Moon and others 1976, Isaacson and others 1978). This discrepancy was possibly due to the comparative insensitivity of the earlier test procedures for K99 antigen detection. In this study the more sensitive minca-Isovitalex isolation method of Guinee and others (1977) was used.

Calf ligated intestinal loops were considered to be the most sensitive method for screening for enterotoxigenicity. The failure of any non-ETEC isolates tested to dilate calf gut loops suggests that the infant mouse assay was of comparable sensitivity, and that other virulence factors which may have been present such as the adhesive antigen F41 (Morris and others 1982) may only occur in conjunction with the K99 and stable toxin characteristics. For these reasons calf ETEC in

Scotland and northern England probably can be diagnosed by detection of K99 antigen alone.

The absence of any positive labile toxin calf ETEC in this survey is in general agreement with results from other surveys (Moon and others 1976, Isaacson and others 1978, Nakazawa and others 1981) but contradicts serological surveys in cows (Whipp and Donta 1976) and calves (Dobrescu 1979) which have found a high proportion of animals to have antibodies against labile toxin. Bacteria other than *E coli* have been shown to elaborate labile toxin (Jiwa and others 1981) and these organisms could account for labile toxin antibodies present in bovine sera. The nature of the non-neutralisable Y1 adrenal cell-rounding factor was not determined, and its role in enteric disease is not known.

Twenty-eight ETEC were examined for the Vero cytotoxic factor. Only one of these isolates was positive, an isolation frequency similar to that reported by Kashiwazaki and others (1980). The role of the Vero cytotoxic factor in the pathogenesis of calf diarrhoea is not known, but its low association with ETEC suggests that its presence may not be significant.

Limited serotyping revealed that ETEC were mainly associated with the O8 serogroup, and all O8 isolates were haemolytic. Haemolysin production by *E coli* has not been correlated with calf ETEC (Smith and Halls 1967a, Sivaswamy and Gyles 1976), nor has it been shown to be a significant virulence character for non-invasive enteropathogenic *E coli* (Linggood and Ingram 1982). In the present survey there was a good but not absolute correlation between the haemolytic character and ETEC, but caution should be exercised in assigning virulence to *E coli* on the basis of haemolysis.

The comparison of ETEC and non-ETEC isolates for their resistance to seven antibiotics showed that, except for neomycin and streptomycin, a higher proportion of non-ETEC isolates were more widely resistant to the antibiotics tested. ETEC isolates from the same farm generally showed similar resistance patterns but between farms the resistance patterns were different. Little information exists on the antibiotic resistance of ETEC, although a previous survey (Sivaswamy and Gyles 1976) showed no difference between ETEC and non-ETEC. There is no evidence to suggest that multiple antibiotic resistant *E coli* cause disease (Linton 1982), therefore this characteristic should not be used as a marker of ETEC in veterinary diagnostic laboratories.

The survey of cow and calf sera for the presence of K99 antibodies confirmed the low prevalence of ETEC. It has been shown that calves seroconvert to K99 antigen after experimental challenge with ETEC (Snodgrass and others 1982a), thus serology could clearly be helpful in identifying herds in which ETEC were present. Previous seroepidemiological studies in Holland (Ellens and others 1978) and Israel (Kornitzer and Tamarin 1979) also showed very few cows and calves to be serologically positive for K99 antigen.

The majority of calves were found to be infected with ETEC when less than 24 hours old, but on two of eight farms some ETEC infected calves were concurrently infected with rotavirus when 24 to 72 hours old. This age susceptibility period is consistent with previous epidemiological studies (Smith and Halls 1967a, Acres and others 1975, Morin and others 1976, Moon and others 1978, Lariviere and others 1979).

Other enteropathogens such as rotavirus, coronavirus and *Cryptosporidium* species were isolated more frequently from diarrhoeic than clinically normal calves, although these differences were only significant overall for rotavirus ($P < 0{\cdot}01$). Including ETEC isolations, only 50 per cent of calves with diarrhoea were shown to be infected with an enteropathogen. A recent survey however (Snodgrass and others 1982b) suggested that a significant association between enteropathogens and diarrhoea can be shown in 80 per cent of calf diarrhoea outbreaks. Other factors such as management, environment, insensitivity of diagnostic tests and possible unrecognised enteropathogens may contribute to diarrhoea in the remaining calves.

In conclusion enterotoxigenic colibacillosis is a major cause of diarrhoea in calves less than three days old but is not associated with the typical diarrhoea problems seen on most farms in calves older than three days (Snodgrass and others 1982b).

Acknowledgements.— We thank the Scottish veterinary investigation centres and Mr B. A. Synge for the collection of faecal samples, the staff of the clinical studies department of the institute for provision of laboratory mice and calves, M. McGeachin for brush border adhesion studies and Drs F. and I. Orskov for serotyping *E coli*. D. Sherwood was in receipt of an Agricultural Research Council studentship and M. McGeachin of a Wellcome veterinary vacation scholarship.

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REPRINTED FROM THE VETERINARY RECORD, AUGUST 16, 1980

Diarrhoea in unweaned piglets associated with rotavirus and coccidial infections

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Veterinary Record (1980) **107**, 156-157

DIARRHOEA in unweaned piglets is widely recognised in the United Kingdom and elsewhere. A variety of names have been used to describe the condition: "non-infective diarrhoea" (Smith and Jones 1963); "milk scours" (Stevens 1963); "nutritional scours" (Jones 1967); "white scours" (Mouwen 1972) and "three week enteritis" (Stevens 1963). *Escherichia coli* infection shortly after birth, transmissible gastroenteritis virus and *Clostridium perfringens* infection have been considered to play a part in the aetiology (Jubb and Kennedy 1970). Recently Bohl (1979) has suggested that rotavirus is frequently associated with this type of diarrhoea.

A scour problem on a pig unit in North East Scotland was investigated in February 1979. The unit consisted of 650 sows farrowing in crates, with piglets on woven mesh flooring except for a solid concrete creep area. Some build up of faeces behind the sows occurred despite the mesh flooring. Piglets were weaned at two weeks of age. For two years outbreaks of diarrhoea had been seen in piglets from one week of age to weaning. The severity of the condition ranged from loose faeces for one to two days only, to persistent diarrhoea for five days or longer with resultant severe dehydration and deaths. Diarrhoeic faeces varied from cottage cheese-like, watery grey and fetid, or yellow and frothy with curd-like flecks. The morbidity within litters and in the farrowing house as a whole also varied considerably. On occasions only some litters had piglets with diarrhoea, but at other times many litters were affected, with a high morbidity in individual litters. As the morbidity increased, so did the severity of diarrhoea and the percentage mortality. Affected piglets were often thinner than non-diarrhoeic litter mates, and became gaunt and hairy in appearance. Response to a wide range of antibiotics was very poor, but some pigs recovered spontaneously while others continued to scour until weaning. If severely affected piglets were not too young, weaning hastened recovery.

Evidence of coccidial infection was first noted in a 10-day old piglet killed within 24 hours of onset of diarrhoea. Although no obvious gross pathological changes were seen at necropsy, histopathological examination revealed extensive villous atrophy and crypt hyperplasia in the small intestine (Fig 1). Many villi were covered by cuboidal epithelium and mitotic figures were numerous in crypts. Developing coccidial

FIG 1: Jejunal villous atrophy in scouring piglet. H&E × 60

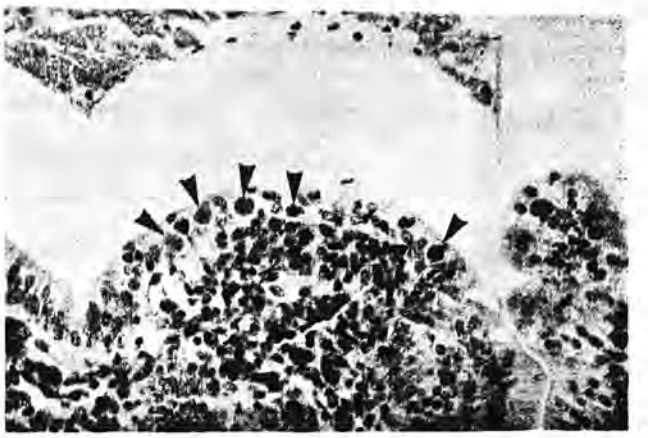

FIG 2: Same piglet, illustrating different stages of coccidia (arrowed) in ileal surface cells. H&E × 370

forms were present within the intestinal epithelium (Fig 2). Rotavirus infection was also demonstrated in the intestinal epithelium by immunofluorescence.

After this initial observation coccidial oocysts were observed in faeces of scouring piglets, especially soon after the onset of diarrhoea. Oocysts were counted using a McMaster worm egg slide (Hawksley, England) numbers varied from 1×10^3 to 1×10^7 per g of faeces. Oocysts (1×10^2 to 9×10^4 per g) were also detected in the faeces of sows within the farrowing house. Sporulation of oocysts was effected in 2·5 per cent potassium dichromate solution and indicated that the coccidium involved was *Isospora suis*.

An extensive search for other potential pathogens was also made. Rotavirus was detected sporadically in the faeces of scouring piglets by counterimmunoelectrophoresis (Middleton and others 1976) though usually at low levels of infection. No evidence was found of infection with enteropathogenic strains of *E coli* in affected litters.

Preliminary observations have indicated that treatment of the sows with amprolium pre-mix (Merck Sharp & Dohme) at 1 kg per ton in feed immediately before entry into and during their stay in the farrowing house reduced oocyst excretion by the sows and the incidence of diarrhoea in their litters.

The problem of diarrhoea in unweaned piglets in this herd resembles that reported widely in the literature. The lesions of villous atrophy and crypt hyperplasia have been described by others (Mouwen 1972). Villous atrophy has been described in porcine rotaviral infection (Pearson and McNulty 1977) and in experimental *I suis* infection (Stuart and others 1980). Workers in North America (Sangster and others 1976 and 1978) have described diarrhoea in unweaned piglets caused by coccidial infection and their findings resemble those reported here. Moreover, Stuart and Lindsay (1979) have recently reported identical changes in experimental piglets dosed orally with *I suis* oocysts, while Bergeland (1977), in a recent survey of necrotic enteritis in nursing piglets, found coccidial infection to be the most common cause.

Coccidial infection has previously not been regarded as important in unweaned piglets in the United Kingdom, but our findings suggest that it should be considered as a potential complicating factor in cases of diarrhoea in this age of pig.

Acknowledgements.—The authors wish to express thanks to the bacteriology department, Ministry of Agriculture Veterinary Laboratory, Lasswade for serotyping of *E coli* isolates.

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Printed in Great Britain by R. W. Simpson and Co. Ltd., 70 Sheen Road, Richmond, Surrey

Papers and Articles

Aetiology of diarrhoea in young calves

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Veterinary Record (1986) **119**, 31-34

Faeces samples were collected from 302 untreated calves on the day of onset of diarrhoea and from 49 healthy calves at 32 farms experiencing outbreaks of diarrhoea. At least four diarrhoeic calves were sampled on each farm, and samples were examined for rotavirus, coronavirus, cryptosporidium, enterotoxigenic *Escherichia coli* and *Salmonella* species. Although all these enteropathogens were excreted more frequently by the diarrhoeic than by the healthy calves, the difference was significant overall only for rotavirus. Rotavirus was excreted by 18 per cent of healthy calves, coronavirus by 4 per cent, cryptosporidium by 14 per cent, and no enterotoxigenic *E coli* or *Salmonella* species were detected. The most common enteropathogen in diarrhoeic calves was rotavirus, which was excreted by more than half the diarrhoeic calves on 18 farms. Coronavirus was excreted at a similar high prevalence on one farm, cryptosporidium on five farms and enterotoxigenic *E coli* on three farms. Concurrent infection with two or more microorganisms occurred in 15 per cent of diarrhoeic calves. There was no difference in the isolation rate of campylobacters between diarrhoeic and healthy calves.

DIARRHOEA in young preweaned calves is a syndrome of great aetiological complexity. In addition to the influence of varied environmental, managemental, nutritional and physiological factors, the infectious agents capable of causing diarrhoea in the neonatal calf are numerous. Thus while reports of the occurrence of individual microorganisms as causes of diarrhoea are common, no surveys of outbreaks of diarrhoea using techniques suitable for detecting a wide range of agents have been conducted in the United Kingdom.

Surveys carried out elsewhere generally show that the most important infectious agents are rotavirus, coronavirus, enterotoxigenic *E coli*, *Salmonella* species and cryptosporidium (Morin and others 1976, Acres and others 1977, Moon and others 1978, Tzipori 1981, Bulgin and others 1982, Moerman and others 1982). These five microorganisms are all known to occur in the United Kingdom (Woode and Bridger 1975, Snodgrass and others 1980, Sherwood and others 1983).

In addition there has been recent interest in campylobacters as potential causes of enteritis in calves (Al-Mashat and Taylor 1980a, b, 1981, 1983a, Firehammer and Myers 1981) although some investigators consider that they are part of the normal enteric flora of ruminants (Florent 1959, El Azhary 1968, Prescott and Bruin-Mosch 1981).

This paper describes a survey carried out in Scotland and the north of England, using diagnostic techniques designed primarily to detect the five principal enteropathogens and campylobacters. The aim was to obtain faecal samples from sufficient numbers of calves to be representative of the enzootic situation on each farm and to allow statistical comparison of the data with data obtained from healthy calves, and to sample sufficient farms to be representative of the wider situation.

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Materials and methods

Farms

Thirty-two farms in Scotland and the north of England were included in this study. There were eight dairy farms, usually with extended calving seasons from late summer through to spring. Management varied considerably but in all cases calves were removed from the cow at not more than two days old and were thereafter reared in individual pens on milk substitutes. The cows were predominantly Friesian/Holstein, with both dairy and beef-breed bulls being used. The calving season on the 24 beef suckler herds was predominantly from autumn to spring, with pronounced seasonal peaks in autumn and late winter/early spring. The majority of scour problems occurred in the latter calving period. Most of the beef herds were housed throughout the winter. The cows were usually traditional dairy/beef crosses (Hereford cross Friesian or shorthorn cross Galloway) and the predominant sire breed was Charolais.

All the farms included in the survey had reported calf diarrhoea problems with a 20 to 100 per cent morbidity.

Calves

The calves from one to 28 days old and at least four diarrhoeic calves were sampled on each farm on the day of onset of diarrhoea before any treatment had been given. Non-diarrhoeic calves of similar age were also sampled, although on farms with high morbidity this was often not possible. The faeces samples were collected into plastic bottles and submitted to the laboratory, usually by post.

Faeces examination

Faeces were examined for rotavirus by electron microscopy (Snodgrass and others 1976) and enzyme-linked immunosorbent assay (ELISA) (Fahey and others 1981); for coronavirus by electron microscopy ELISA and haemadsorption elution haemagglutination assay (van Balken and others 1979); and for cryptosporidium by faecal smears stained with Giemsa (Snodgrass and others 1980). Faeces were cultured on blood agar and McConkey agar; coliforms were subcultured on Minca-Isovitalex agar before testing for K99 antigen by slide agglutination with specific antisera (Sherwood and others 1983). The presence of K99 was taken as evidence of the enterotoxigenicity of *E coli* isolates (Sherwood and others 1983). *Salmonella* species were detected by routine techniques after overnight enrichment in sodium selenite broth.

Campylobacters were isolated by direct culture of faeces on Preston medium (Bolton and Robertson 1982) incorporating 100 iu/ml of nystatin and FBP compound (Oxoid Ltd) as modified by Skirrow and Benjamin (1980a). Plates were placed in anaerobic jars without catalyst, evacuated to −650 mmHg, and hydrogen added to atmospheric pressure; then 10 per cent of the hydrogen was replaced by carbon dioxide. Cultures were incubated at 37°C for seven days and campylobacter-like colonies were subcultured on to Columbia blood agar plates (Oxoid). Campylobacters were classified as *C jejuni*, *C coli*, *C fetus* or *C hyointestinalis* (Skirrow and Benjamin 1980a, b, Karmali and others 1980, 1981, Gebhart and others 1983, Terzolo 1984).

Statistical analysis

Corrected χ^2 analysis was used to compare the prevalence of enteropathogens in diarrhoeic and healthy calves. The significance of association between individual microorganisms was also examined by χ^2 analysis.

Results

Occurrence of enteropathogens in healthy calves

Faeces samples were obtained from 49 healthy calves on 14 farms. Rotavirus was detected in nine calves, coronavirus in two calves, cryptosporidium in seven calves. No enteropathogenic *E coli* species, *Salmonella* species or mixed infections of these organisms were detected (Table 1).

Occurrence of enteropathogens in diarrhoeic calves

Faeces samples were also examined from 302 diarrhoeic calves at 32 farms (Table 2). Rotavirus was detected in 152 (50 per cent) calves in total, and in 45 of these calves the rotavirus was present in combined infections with either or both coronavirus and cryptosporidium. The other enteropathogens were detected at lower prevalences; cryptosporidium (25 per cent), coronavirus (8 per cent) enterotoxigenic *E coli* (4 per cent) and *Salmonella* species (1 per cent). In total, infection was detected in 216 of 302 diarrhoeic calves (72 per cent); concurrent infection with two microorganisms occurred in 45 calves (15 per cent) and with three microorganisms in one calf (0·3 per cent).

TABLE 1: Prevalence of enteropathogens on 14 farms where both diarrhoeic and healthy calves were examined

Enteropathogen	Number of positive samples/ number of calves examined		Significance (P)
	Diarrhoeic calves	Healthy calves	
Rotavirus	66/128	9/49	<0·001
Coronavirus	16/128	2/49	NS
Cryptosporidium	31/128	7/49	NS
Enterotoxigenic *E coli*	8/109	0/49	NS
Salmonella species	2/109	0/49	NS

NS Not significant (P>0·05)

TABLE 2: Detection of enteropathogens in 302 diarrhoeic calves on 32 farms

Enteropathogen	Number of calves
Rotavirus	107
Coronavirus	11
Cryptosporidium	39
Enterotoxigenic *E coli*	11
Salmonella species	2
Rotavirus + cryptosporidium	33
Rotavirus + coronavirus	11
Coronavirus + cryptosporidium	1
Rotavirus + coronavirus + cryptosporidium	1

TABLE 3: Prevalence of enteropathogens in diarrhoeic calves on outbreak farms. The comparative figures for healthy calves were that rotavirus was excreted by 18 per cent, coronavirus by 4 per cent, cryptosporidium by 14 per cent and enterotoxigenic *E coli* by 0 per cent

Enteropathogen	Number of farms in each range (%) of enteropathogen detection rate				
	0	1–24	25–49	50–74	75–100
Rotavirus	7	1	6	10	8
Coronavirus	19	7	5	1	0
Cryptosporidium	8	11	8	4	1
Enterotoxigenic *E coli**	27	0	1	2	1

* Calves were tested from 31 farms only

TABLE 4: Prevalence of campylobacters in diarrhoeic and healthy calves

Species	Number of positive samples (%)	
	Diarrhoeic (n=156)	Healthy (n=34)
C jejuni	34 (22)	10 (29)
C hyointestinalis	14 (9)	4 (12)
C coli	1 (0·5)	0
C fetus	1 (0·5)	0
Total	50 (32)	14 (41)

The data were analysed to detect any correlations between the occurrence of different infectious agents in individual animals. No positive correlations were detected, but there was a significant negative correlation between the occurrence of rotavirus and enteropathogenic *E coli* (P<0·01); concurrent infection with these two agents was never detected.

Occurrence of enteropathogens on farms

Enteropathogens were detected on all 32 farms; rotavirus on 25 (78 per cent), cryptosporidium on 24 (75 per cent), coronavirus on 13 (41 per cent), enterotoxigenic *E coli* on four (13 per cent) and *Salmonella* species on two (6 per cent). Two enteropathogens were detected on 12 farms (most commonly rotavirus and cryptosporidium on nine farms [28 per cent]) and three enteropathogens were detected on 12 farms (most commonly rotavirus, coronavirus and cryptosporidium on 11 farms [34 per cent]).

A comparison was made of the prevalence of the different agents in diarrhoeic and healthy calves on the 14 farms from which samples were obtained from both groups (Table 1). Although rotavirus, coronavirus, cryptosporidium, and enterotoxigenic *E coli* were all detected more frequently in diarrhoeic than in normal calves, the difference was statistically significant only for rotavirus (P<0·01).

The ranges of prevalence for each enteropathogen during each farm outbreak were tabulated and compared (Table 3). Rotavirus was particularly common, ie, more than half the diarrhoeic calves excreted rotavirus on 18 farms (56 per cent), more than half excreted coronavirus on one farm (3 per cent), cryptosporidium on five farms (16 per cent) and enterotoxigenic *E coli* on three farms (9 per cent).

Occurrence of campylobacters

Campylobacters were isolated from 14 of 34 healthy calves (40 per cent) and from 50 of 156 diarrhoeic calves (31 per cent) (Table 4). There was no significant difference between diarrhoeic and healthy calves in the prevalence of the different species or of all campylobacters. They were isolated from calves on 16 of 21 farms investigated.

All the diarrhoeic calves from which enterotoxigenic *E coli* were isolated were less than six days old, and most of them were only one to two days old. The diarrhoea was watery and the calves rapidly became dehydrated. Their response to

appropriate antibiotic therapy was good and antibiotics were subsequently used prophylactically for the first three days of life. The calves which recovered thrived.

The other infectious agents were generally part of a syndrome of diarrhoea in calves from four days to four weeks old, most typically from five to 10 days. On one farm with particularly bad hygiene, diarrhoea in one-day-old calves was caused by rotavirus.

Morbidity was generally 50 to 100 per cent in the groups of calves affected. Mortality varied from 0 to 30 per cent, and the most severe outbreaks were caused by rotavirus either alone or in combination with coronavirus or cryptosporidium. On these farms substantial setbacks to the subsequent growth of the calves often occurred. A subjective impression was formed that cryptosporidiosis was generally less clinically severe. Calves infected with cryptosporidium alone had a low mortality, usually recovered without supportive therapy and grew normally after recovery.

Discussion

In this survey faeces samples from four or more calves from each outbreak of diarrhoea were examined for the presence of five common enteropathogenic organisms. At least one agent was detected on each farm and more usually two or three infectious agents were present. Ascertaining the role played by each agent is thus difficult.

The role of the bacterial enteropathogens was comparatively easy to define. Enterotoxigenic *E coli* apparently caused a relatively infrequent but significant and clinically distinct diarrhoea in one to two-day-old calves. This restriction of enterotoxigenic *E coli* to the youngest calves has been noted by others (Morin and others 1976, Acres and others 1977, Moon and others 1978, Bulgin and others 1982, Moerman and others 1982) and has also been observed experimentally (Smith and Halls 1967). Salmonellosis did not cause any of the problems investigated. If the survey had included calves bought through markets in addition to home-bred calves, a significant involvement for *Salmonella* species could have been expected (Wray and Sojka 1977). The results failed to demonstrate any link between diarrhoea and the faecal isolation rate either of all campylobacters or of one particular species. This supports suggestions that campylobacters are common in both healthy and diarrhoeic calves (El Azhary 1968, Prescott and Bruin-Mosch 1981). However, it is possible that they may be of more significance in enteritis of older, ruminating calves (Al-Mashat and Taylor 1980b, 1981, 1983a).

The role of the non-bacterial agents was more complex, because they were associated with similar disease in similar age ranges and frequently coinfected the same calf. Of these three agents, the role of rotavirus was easiest to assess. It was apparently the dominant enteropathogen, being excreted by more than half the diarrhoeic calves on 56 per cent of the farms. Most other surveys have also shown rotavirus to be of major importance (Morin and others 1976, Moon and others 1978, de Leeuw and others 1980), although Bulgin and others (1982) found that rotavirus was comparatively uncommon. Our subjective impression that rotavirus was associated with the severest clinical problems suggests a parallel with the human disease; children with rotavirus diarrhoea were significantly more likely to require treatment and to become dehydrated than those with diarrhoea caused by enterotoxigenic *E coli* (Black and others 1981). Similarly the unthriftiness after recovery observed in some rotavirus-infected calves is consistent with the observation of prolonged deficiencies in nutrient intake and absorption in rotavirus-infected children (Molla and others 1982).

The role of coronavirus remains enigmatic. In North America coronaviruses are considered to be important causes of calf diarrhoea (Morin and others 1976, Moon and others 1978), but they have never been shown to be common enteropathogens in the United Kingdom. It is possible that they are of more significance in other conditions such as winter dysentery of adult cattle (Takahashi and others 1980, Espinasse and others 1982).

Cryptosporidiosis occurred in more than 50 per cent of the diarrhoeic calves on five farms, and more than 10 per cent of all scouring calves excreted cryptosporidium at the same time as rotavirus. Our observations on the relative mildness of cryptosporidiosis in calves have been supported by Bulgin and others (1982). Experience with a rotavirus/K99 *E coli* vaccine (Snodgrass and others 1982a) in the field suggests that the most common cause of diarrhoea after successful vaccination for rotavirus and enterotoxigenic *E coli* is cryptosporidium (Snodgrass, unpublished information). However, specific prophylactic or therapeutic controls for cryptosporidiosis are still lacking.

Mixed infections occurred in 15 per cent of diarrhoeic calves. However, the pathogenic significance of these is not clear. The most common combination was rotavirus and cryptosporidium and no interaction of these two agents has been shown experimentally (Tzipori and others 1981). Although significant interactions have been demonstrated experimentally between rotavirus and enterotoxigenic *E coli* (Gouet and others 1978, Runnels and others 1980, Snodgrass and others 1982b) this combination did not occur in any calf in this survey.

Although enteropathogenic organisms were detected on all the farms investigated, on some farms none of these agents was particularly common. This emphasises the difficulties in investigating and diagnosing the infectious aetiology of calf diarrhoea outbreaks, even in surveys such as this where relatively large numbers of calves were sampled. Although the five infectious agents specifically included in this survey are certainly important and enteropathogenic, other microorganisms which are at present not known to be important may also have to be included in future surveys.

New pathogenic mechanisms continue to be described for *E coli*, with verocytotoxic strains being detected in calves (Sherwood and others 1985), and with the detection of F41 and Att25 adhesins (Morris and others 1980, Lintermans and Pohl 1983). There are many other enteric bacteria potentially associated with disease in calves (Al-Mashat and Taylor 1983b). Other enteric viruses such as astrovirus, calicivirus-like agent and Breda virus may also prove to be important (Woode and others 1982, Bridger and others 1984), and calicivirus-like viruses have been detected by the authors in three calves not connected with this survey (Snodgrass and Menzies, unpublished information).

The results from this survey and the broadly similar results from a parallel survey in the south of the United Kingdom (Reynolds and others 1986) provide a rational starting point for planning control measures and suggest that effective vaccines to control rotavirus infection would be likely to be of considerable benefit in the overall control of diarrhoea in young calves.

Acknowledgements. — This survey was made possible by the goodwill and cooperation of many farmers, their veterinary surgeons and veterinary investigation officers, all of whom we thank.

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JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1982, p. 473–477
0095-1137/82/090473-05$02.00/0

Vol. 16, No. 3

Rapid Diagnosis of Rotavirus Infection by Direct Detection of Viral Nucleic Acid in Silver-Stained Polyacrylamide Gels

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Received 29 March 1982/Accepted 7 June 1982

A rapid simple technique for the diagnosis of rotavirus has been developed based on the sensitive detection of rotavirus double-stranded RNA genome segments separated in polyacrylamide gels. The method utilizes a recently described ultrasensitive silver stain for polypeptides, which can also detect subnanogram amounts of nucleic acid. The sensitivity of the technique is comparable with that of electron microscopy or enzyme-linked immunosorbent assay.

Rotaviruses cause enteritis in a wide variety of species and have been intensively investigated for more than a decade (reviewed 5, 14). As members of the *Reoviridae* (13), they have a double-stranded RNA (dsRNA) genome consisting of 11 segments ranging in molecular weight from approximately 2.0×10^6 to 0.2×10^6 (9). Electrophoretic analysis has revealed major differences in the mobility of the genome segments between virus isolates from different host species and minor differences between individual isolates from the same species. Thus, genome electropherotyping has been the most common method for both taxonomic and epidemiological studies (9, 12, 18).

Recently, several methods have been reported which utilize silver staining for the ultrasensitive detection of polypeptides resolved by polyacrylamide gel electrophoresis (PAGE) (16, 17, 20). We used one of these methods (20) to monitor the purification of rotavirus from feces and found that the dsRNA bands were also stained with high sensitivity. This result was consistent with the well-described property of silver ions to form a stable complex with nucleic acids (7). Similar nucleic acid staining with another silver staining method has recently been reported by Somerville and Wang (22).

In this communication we describe a diagnostic test for rotavirus in feces based on this ultrasensitive detection of viral dsRNA, which has the advantages of simplicity, economy, and speed, and which simultaneously identifies the electropherotype.

MATERIALS AND METHODS

Fecal specimens. Infected and control fecal specimens were obtained from cattle and human sources. The bovine samples and two of the human samples were examined for rotavirus by electron microscopy (EM) and by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially by the method of Yolken et al. (25), using a hyperimmune rabbit serum raised against tissue culture-grown bovine rotavirus, with a neutralization titer of 1:10,240 both to coat the wells and as a conjugate to detect antigen. Unconcentrated samples were examined by EM as described by Snodgrass et al. (21). The samples in the dilution experiment were coded and scored blind. The majority of the human specimens were kindly provided by the Edinburgh Regional Virus Laboratory. Rotavirus diagnosis had been carried out on these specimens by the cell culture method of Bryden et al. (2).

Nucleic acid extraction. Fecal samples were diluted 1:4 by weight with 0.1 M sodium acetate buffer (pH 5.0) containing 1% (wt/vol) sodium dodecyl sulfate; the normal sample size used was 0.25 g of feces, which provided enough extract for at least 10 separate analyses. An equal volume of a 3:2 (vol/vol) 'phenol'-chloroform mixture was added to the fecal suspension, and the sample was mixed for 1 min. ('Phenol' consisted of a mixture of 500 g of phenol, 70 g of *m*-cresol, and 200 g of water containing 0.5 g of 8-hydroxyquinoline.) The emulsified mixture was then centrifuged for 10 min at $1,200 \times g$, and the resulting clear upper aqueous layer was removed. A sample was then prepared for electrophoresis by the addition of 10 μl of 25% (wt/vol) sucrose containing 0.2% bromophenol blue to 40 μl of the aqueous layer.

Occasional samples failed to yield sufficient clear aqueous layer, but it was found that either further centrifugation for 3 min at $16,000 \times g$ in a microcentrifuge (Mechanika Precyzyjna, type 320a) or the addition of 0.5 ml of buffer followed by remixing and centrifugation at $1200 \times g$ gave an ample clear layer.

PAGE. The 50-μl samples were loaded onto 5% polyacrylamide slab gels (acrylamide-to-bis-acrylamide ratio of 37.5:1) which were polymerized with 0.01% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine and 0.05% (wt/vol) ammonium persulphate. The gel and electrode buffer was 0.036 M Tris–0.03 M

sodium dihydrogen phosphate–0.001 M EDTA (pH 7.8). Gel dimensions were 14-cm wide by 19-cm long and 0.15-cm thick. It should be noted that the gel thickness is critical with the silver staining technique (20). Deep sample wells (0.6 by 2.0 cm) facilitated loading without the transfer of sample to neighboring wells. Electrophoresis was performed at room temperature for 16 h at 20 mA and 70 V. In most experiments, one of the glass plates used to form the gel mold was treated with a 1% solution of Silane 174A in ethanol (Union Carbide Corp.) for 10 min, dried in air, rinsed in distilled water, and redried. This treatment caused the gel to adhere strongly to the plate and greatly simplified its handling during staining.

Silver staining. The gels were stained by using a slight modification of the method of Sammons et al. (20). The initial fixation steps described for protein staining were omitted, and the gels were washed with 10% ethanol–0.5% acetic acid for 30 min and then soaked in 0.011 M silver nitrate for 2 h. The gel was then rinsed briefly in distilled water, and the reduction step was performed with a solution of 0.75 M sodium hydroxide containing 0.1 M formaldehyde and 0.0023 M sodium borohydride. The bands appeared at this stage, and the reduction was continued until the bands were clearly visible for a maximum of 10 min. In our early experiments the gels were then placed in 0.07 M sodium carbonate, and the intensity of staining of both the bands and the background increased slightly in the 20 min or so after transfer. After 30 min, the gels were placed in fresh carbonate solution. However, it was found that, when using gels which were stuck to a glass plate with Silane 174A, an unacceptable degree of background staining sometimes developed when the gel was placed in the carbonate solution. This could be prevented by treating the gel with a 5% acetic acid solution for 30 min after the reduction and then transferring the gel to carbonate solution for storage. Gels have been successfully stored, sealed in polythene bags, for up to 6 months.

All of the solutions for the staining were made from single distilled water and, with the exception of the initial fixation solution, were degassed before use. The solutions were used in 200-ml volumes in a single plastic staining dish, and care was taken to avoid touching the gel surface with ungloved hands. Constant agitation of the solutions throughout the procedure was achieved with a rocking bed destainer. The gels were photographed by transmitted light, using a Wratten 85B filter.

Purification of virion dsRNA. Virus was purified from infected bovine feces essentially by the method described by Todd and McNulty (24), and the dsRNA was extracted with phenol and further purified by one cycle of CF11 cellulose chromatography (6) performed as described by Bevan et al. (1). The resulting dsRNA was quantified spectrophotometrically.

RESULTS

The results obtained by direct extraction of feces with phenol followed by gel analysis of the extract are shown in Fig. 1. The first nine samples were all from a herd affected with enteritis, and eight may be clearly seen to contain the characteristic dsRNA segments of bovine rotavirus; the other three control samples were negative. As would be expected, the samples from a single outbreak all showed the same electropherotype. Other bands were seen on the gels, especially near the origin where DNA forms a diffuse band, and occasional samples produced a continuous smear of stained material; but neither of these effects interfered with the detection of the dsRNA. The rotaviral genome segments could be identified by the characteristic sharpness and unique pattern of the dsRNA bands.

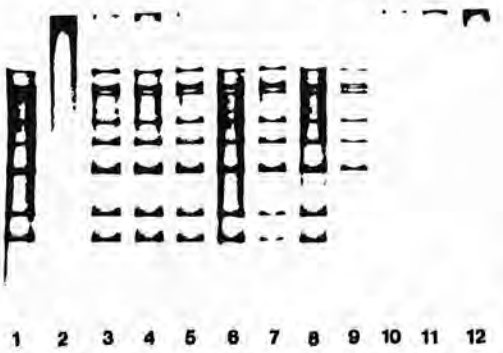

FIG. 1. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 9, extracts of fecal samples from diarrheic calves; tracks 10 through 12, extracts of feces from uninfected calves.

The results of a comparison between PAGE, ELISA, and EM are summarized in Table 1, together with the results of PAGE analysis on 24 human specimens which had been tested for rotavirus by cell culture. There was complete concordance between PAGE and ELISA results and only a single conflicting result in the PAGE and EM results. The level of virus in this one sample was clearly low, as the dsRNA bands were faint. The results with the human samples similarly showed just one conflicting result, which was positive by PAGE; this sample was obtained from a patient who also yielded other samples which were positive by cell culture.

The sensitivity of PAGE was investigated by dilution experiments. A positive sample judged to contain an average level of viral dsRNA was serially diluted with a negative sample to give a range of viral concentrations from 12.5 to 0.2% of that in the original sample, but with approximately the normal amount of contaminating nonviral material in each dilution. Extracts of these samples were analyzed by PAGE, and the results are shown in Fig. 2. Rotavirus dsRNA segments 1 through 4 were detected in dilutions down to 0.39%, but the lower-molecular-weight bands were not apparent at the higher dilutions. Figure 2 also shows the result of diluting the positive sample extract with electrophoresis

TABLE 1. Comparison of PAGE with other methods of rotavirus diagnosis

Specimen	No. of samples	Diagnostic method[a]				No. of samples in category
		PAGE	ELISA	EM	Cell culture	
Bovine	68	+	+	+	ND	41/68
		−	−	−	ND	26/68
		+	+	−	ND	1/68
Bovine	13	+	+	ND	ND	5/13
		−	−	ND	ND	8/13
Human	2	+	+	+	ND	1/2
		−	−	−	ND	1/2
Human	24	+	ND	ND	+	18/24
		−	ND	ND	−	5/24
		+	ND	ND	−	1/24

[a] +, Positive; −, negative; ND, test not performed.

buffer. All of the dsRNA segments were detected at a level of 0.39% of the original positive material.

A similar dilution series, using the same positive and negative feces and the appropriate buffers, was constructed and tested by ELISA and by EM (Table 2). EM was found to detect virus to a level of 1.56% of the original sample. The ELISA results are expressed as ratios of the positive and negative optical densities (P/N) as suggested by Yolken et al (25), who considered any value in excess of 2.1 to be rotavirus positive. Table 2 shows two values of P/N for each dilution. The first, and higher value, was based on the optical density given by the particular negative feces used for dilution, and the second is based on the optical density given by our standard uninfected feces. These data show the dilution series positive to the 0.39% and 1.56% levels, respectively. Thus, with the methods used, the sensitivities of PAGE, EM, and ELISA were approximately equal.

One further dilution series was investigated to determine the sensitivity of the silver staining method in absolute terms. Examination of gels loaded with a dilution series of purified dsRNA showed that the detection limit of the silver stain for a single band was 300 to 400 pg.

DISCUSSION

The diagnosis of rotavirus infection has been achieved by a variety of methods (reviewed in 10) based on either the direct visualization of the virion by EM or the detection of viral antigens by a wide diversity of immunological techniques, including the highly advanced and sensitive enzyme-linked fluorescence assay (26). The test described above is based on the direct extraction and detection of viral dsRNA. Two direct extraction methods have been reported previously, but both have been designed for epidemiological studies and genome analysis rather than for diagnosis. Clarke and McCrae (3) described a method based on end labeling of total fecal nucleic acid followed by CF11 cellulose purification of the dsRNA and analysis by PAGE and autoradiography. This procedure could detect rotavirus dsRNA with very high sensitivity, but is too protracted and expensive to use as a routine diagnostic test. Theil et al. (23) recently reported a method for the bulk extraction of dsRNA from large fecal samples (6 ml), using CF11 cellulose as a batch procedure. Their results confirmed that directly extracted dsRNA was identical to virion dsRNA. It is an indication of the increased sensitivity of the silver staining method relative to ethidium bromide fluorescence that Thiel et al. (23) used the dsRNA from 0.4 ml of feces for a single analysis, whereas we routinely use the nucleic acid from only 0.01 ml of feces.

The greatest advantages of the PAGE and silver stain method are its lack of ambiguity and

FIG. 2. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 7, extracts of a dilution series of bovine feces containing 12.5, 3.13, 1.56, 0.78, 0.39, 0.2, and 0% of positive sample, respectively; tracks 8 through 11, four dilutions in buffer of the nucleic acid extract of the positive feces containing 0.39, 0.78, 1.56, and 3.13% of the positive extract, respectively.

TABLE 2. EM and ELISA results on the dilution series of positive feces[a]

Positive feces in dilution (%)	Test		
	EM	ELISA	
		P/N [1]	P/N [2]
12.5	+ [1]	9.12 (+)	4.8 (+)
6.25	+ [16]	5.55 (+)	2.92 (+)
3.13	+ [1]	4.79 (+)	2.52 (+)
1.56	+ [2]	4.25 (+)	2.23 (+)
0.78	−	3.53 (+)	1.85 (−)
0.39	−	2.28 (+)	1.20 (−)
0.20	−	1.86 (−)	0.98 (−)
0	−	1.0 (−)	0.53 (−)

[a] The figures in brackets indicate the number of viral particles found in a standard 10-min search. Positive-to-negative (P/N) ratios are explained in the text. The test results (+, −) are shown in parentheses.

the fact that it provides information about viral electropherotype. Since the test detects the viral genome which has a unique number and pattern of dsRNA segments the results are unequivocal. None of the samples we have examined to date has given any spurious bands which could be confused with viral dsRNA. The only problem encountered in our early trials of the technique was the accidental transfer of sample to a neighboring well in the gel at the time of loading; certainly care is required at this stage, and accurate loading is facilitated by the use of deep sample wells. If confirmation of a weak positive result is required, the sample may be concentrated very simply by ethanol precipitation.

The unambiguous nature of a positive PAGE test contrasts with the difficulties in the interpretation of low-positive values in the ELISA. False-positive results have been reported to occur with ELISA (27), and it has been necessary to incorporate pretreatment of the samples with mild reducing agents (27) or additional controls with blocking antiserum (25), as is our practice.

The fact that the PAGE and silver stain method simultaneously produces an electropherotype is a feature which considerably enhances its value. In recent studies with a human virus the two distinct patterns which are seen for segments 10 and 11 appear to correlate with two major neutralization subgroups of the virus (4, 8). In addition, other major surveys of human viral genome electropherotypes have revealed considerable minor variations (12, 18), and one study has suggested that isolates from neonates may be distinct (18). Lourenco et al. (12) noted the limitation that their clinical samples were too small to allow multiple electrophoretic analyses. Silver staining should permit far more economical use of the dsRNA and has the added advantage that the low loadings required enhance the resolution obtained. The adoption of the gel method for diagnosis should lead to a rapid increase in our understanding of rotavirus epidemiology. The method avoids the problems posed by the recent discoveries of rotaviruses without the group antigen (15, 19) and may also reveal whether the virus can cross species barriers as was recently suggested by McNulty et al. (15).

Most of the samples we have studied to date would be suitable for electropherotype analysis without further purification, but those which give high backgrounds could be conveniently purified by CF11 chromatography (1, 6). The gel system we describe above was selected to allow rapid staining of the gel after electrophoresis and not for maximum resolution of the dsRNA. Discontinuous buffer system gels (11) give the best resolution (18), but such gels require the full fixation and washing procedure described by Sammons et al. (20) to remove sodium dodecyl sulfate. Gels containing agarose cannot be stained by this method.

The method has several other minor advantages. The initial phenol extraction is both virucidal and bacteriocidal and thus eliminates the biohazard and much of the unpleasantness associated with fecal samples. The apparatus and chemicals employed are relatively inexpensive, and there is no dependance on immunological reagents which are variable and expensive to purchase or prepare.

The use of silver staining to detect nucleic acids in such low amounts should have considerable application in the study and diagnosis of other viruses, but it is particularly applicable to dsRNA because of the very sharp bands formed by this species in PAGE and because of the ease with which it may be purified from complex mixtures by CF11 cellulose chromatography (6). We have already found the method most useful for the detection of DNA in velocity sedimentation experiments and for the analysis of small restriction enzyme fragments.

ACKNOWLEDGMENTS

We thank J. M. Inglis and M. F. Jamieson of the Edinburgh Regional Virus Laboratory for supplying the human fecal specimens, Union Carbide Corp. for their kind gift of Silane 174A, P. J. Richardson for his capable technical assistance, and B. J. Easter for photographing the gels.

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EXPERIMENTAL ROTAVIRUS INFECTION IN LAMBS

By

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INTRODUCTION

Reovirus-like agents, for which the name rotavirus has been proposed (Flewett, Bryden, Davies, Woode, Bridger and Derrick, 1974) have been associated with enteritis in the young of several species of animals, and in man. The disease has been reproduced experimentally by the administration of rotavirus to calves (Mebus, Stair, Underdahl and Twiehaus, 1971), piglets (Woode and Bridger, 1975) and mice (Much and Zajac, 1972), and the association of rotaviruses with enteritis in human infants is unequivocal (Davidson, Bishop, Townley, Holmes and Ruck, 1975). A rotavirus has been isolated from lambs with enteritis (Snodgrass, Smith, Gray and Herring, 1976), and this paper records the production of disease in lambs experimentally infected with this virus, and subsequent observations on the lambs.

MATERIALS AND METHODS

Lambs. Six gnotobiotic lambs were used. Three infected lambs were maintained together in one isolator, and 3 controls in another isolator. At 3 weeks of age they were transferred to conventional animal accommodation.

Cell cultures. Secondary calf kidney (CK) and foetal lamb kidney (FLK) cell cultures were grown in Hank's medium containing 10 per cent. adult bovine serum, 10 per cent. lactalbumin hydrolysate (LAH), and 1 per cent. glucose, with penicillin, streptomycin, neomycin, and fungizone, at 300 iu./ml., 300 µg./ml., 16 iu./ml. and 2 µg./ml. respectively. Maintenance medium was Medium 199 with 2 per cent. LAH, 0·5 per cent. bovine serum albumin and antibiotics. Cultures were infected by inoculating virus into the maintenance medium overlaying the cells.

Virus. The virus used for these experiments was originally isolated and described by Snodgrass *et al.* (1976). The isolate did not multiply in FLK or CK cells, but was successfully cultivated in a newborn lamb housed in isolation. A filtrate prepared from 1 g. of intestinal contents was used to infect each experimental animal. The filtrate was prepared by diluting intestinal contents to 20 per cent. in distilled water and filtering through a 0·45 µm. membrane. In addition to this a culture-adapted calf rotavirus was obtained from Dr G. N. Woode (Bridger and Woode, 1975).

Infection of lambs. Three 1-day-old lambs were each infected orally with the filtrate described above and 3 others served as uninoculated controls. All 6 lambs were infected orally with the filtrate at 12 days old.

Observations. The lambs were observed for clinical signs, initially at intervals of not more than 8 h. Faeces samples, collected twice daily for 2 days after inoculation and subsequently daily, were examined by electron microscopy (EM) as described previously (Snodgrass *et al.*, 1976).

Serology. Serum samples were collected before and at intervals after infection, and were titrated by complement fixation (CF), indirect immunofluorescence (IF), and

serum neutralization (SN) techniques. The CF test was performed by the technique described by Grist, Ross, Bell and Stott (1966), with antigen prepared from the calf virus. The antigen was prepared by freezing and thawing CK cultures 6 days after inoculation with virus, and concentrating the harvest 20-fold in ultrafiltration cells through an XM-100A membrane (Amicon) under gas pressure of 0·7 kg./cm² (Kapikian, Cline, Mebus, Wyatt, Kalica, James, Van Kirk, Chanock and Kim, 1975).

The IF test antigen was either lamb or culture-adapted calf rotavirus grown in CK cells on coverslips. The cells were infected with calf virus at a dilution of 10^{-2} and were harvested after 3 days, or with lamb virus as a faecal filtrate inoculated at $10^{-1.5}$ dilution and harvested after 1 day. The antigen was stained with lamb serum followed by anti-sheep globulin conjugated with fluorescein isothiocyanate.

In the SN test, 30 to 300 50 per cent. tissue culture infective doses of lamb or calf rotavirus were mixed with equal volumes of a 10^{-1} dilution of antiserum, and allowed to react at room temperature for 60 min. Virus-antiserum mixtures were then inoculated into CK coverslip cultures, and incubated at 37 °C. Cultures were incubated for 1 day with lamb virus antigen, and for 6 days where antigen was calf virus. The coverslips were read by fluorescence after 1 or 6 days as appropriate, using rabbit antiserum to lamb rotavirus, and sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate.

Antisera were prepared in rabbits by inoculating rotavirus recovered from lamb faeces by centrifugation at 130 000 **g** for 45 min., followed by centrifugation through 40 per cent. sucrose at 130 000 **g** for 4 h. Each rabbit was given an intramuscular inoculation of 2 ml. of an equal mixture of virus suspension and Freund's complete adjuvant followed after 3 weeks by a second intramuscular injection of virus suspension in Freund's incomplete adjuvant.

Detection of latency. Four months after initial infection, the 5 surviving sheep were injected with betamethasone (Betsolan, Glaxo) by intramuscular injection at 1 mg./kg. daily for 5 successive days, in an attempt to provoke rotavirus excretion by immunosuppression. Faeces samples were collected and examined by EM.

RESULTS

Clinical Response

Clinical signs developed 11 to 18 h. after infection of the day-old lambs. All 3 lambs developed brown liquid faeces, and 2 also showed moderate abdominal tension and discomfort on palpation. Anorexia was marked, the mean voluntary milk intake of the infected lambs during the period 9 to 44 h. after inoculation was 780 ± 140 ml., which was significantly less than the 1190 ± 10 ml. taken by the controls ($P < 0{\cdot}01$). Pyrexia was not observed. Although diarrhoea continued for 3 to 4 days, the lambs were otherwise normal by 48 h. after infection. The 3 controls remained normal.

All 6 lambs infected when 12 days old showed mild diarrhoea with no anorexia or other clinical response. Control and recovered lambs were equally affected, and diarrhoea lasted 2 to 3 days.

Virus Excretion

All EM examinations were performed without the microscopist knowing the source of the sample. The 3 lambs infected at one day old excreted rotavirus within 24 h. of infection, and continued to excrete virus for 6 to 7 days. No virus was detected in the faeces of the controls. When infected at 12 days of age, the

previous controls all excreted rotavirus for 1 to 2 days only, while no virus excretion was detected from the recovered lambs.

Serological Response

All animals developed serum antibodies to calf rotavirus after infection as measured by CF and IF tests (Fig. 1). Peak titres were reached after 4 to 6 weeks, and then declined rapidly. The increase in antibody titre detected by both these tests that occurred 3 months after primary infection may have been due to an otherwise inapparent reinfection, as all animals were by this stage in conventional accommodation, allowing indirect contact with other sheep and cattle.

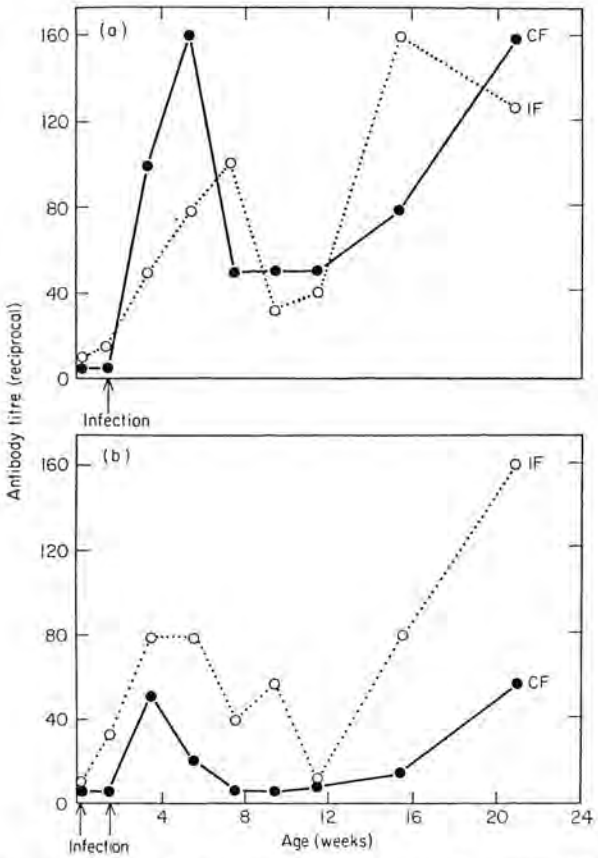

Fig. 1. Serological response of 6 lambs to rotavirus infection. (a) Mean titre of 3 lambs infected at 12 days. (b) Mean titre of 3 lambs infected at 1 and 12 days. CF—complement fixation test titre; IF—immunofluorescent test titre.

A serological response was also demonstrated in the IF test with lamb rotavirus as antigen. However, it was impracticable to prepare sufficient amounts of this antigen to use routinely.

In the SN test, sera from the lambs 3 weeks after infection were screened at

10^{-1} dilution against both calf and lamb virus. No neutralizing antibodies to calf rotavirus were detected. However, a marked reduction in the number of fluorescent cells with lamb rotavirus was evident, a few foci only being present.

Latent Infections

Virus excretion from the lambs was not detected during or after immunosuppressive treatment.

DISCUSSION

The development of clinical disease in lambs following inoculation with lamb rotavirus confirms the pathogenicity of this virus. The relatively mild disease with rapid recovery that was observed is characteristic of that produced by rotaviruses in human infants (Shepherd, Truslow, Walker-Smith, Bird, Cutting, Darnell and Barker, 1975) and calves (Woode, Bridger, Hall and Dennis, 1974). However, it is probable that under field conditions such an infection in a neonate would be greatly exacerbated by stress and bacterial infections.

Twelve-day-old lambs were less susceptible than 1-day-old lambs, as indicated by the absence of anorexia and shorter period of virus excretion. In contrast, calves have been found to be fully susceptible until 8 weeks of age (Woode and Bridger, 1975). It is important to note that virus excretion continued for several days after clinical recovery, which indicates that a clinically normal lamb may be a source of virus infection for other lambs.

Recovered lambs were resistant to virus multiplication when challenged. The mild diarrhoea that occurred in recovered and control lambs at 12 days of age may have been due to the infection, or to the prolonged feeding of a wholly liquid diet.

The use of complement fixation and immunofluorescent serological techniques with calf rotavirus antigen proved satisfactory for demonstrating antibodies to lamb rotavirus. This confirms and extends the group-specific nature of these tests for rotaviruses, as noted by Kapikian *et al.* (1975) and Flewett *et al.* (1974). However, sera from the recovered lambs did not neutralize calf rotavirus, and appeared to only partially neutralize lamb virus. This contrasts with sera from recovered calves, in which high titres of neutralizing antibody to calf rotaviruses can be detected (Mebus, White, Bass and Twiehaus, 1973; Bridger and Woode, 1975). Antisera to rotaviruses from man and pig have been shown to neutralize calf rotavirus to varying extents (Bridger, Woode, Jones, Flewett, Bryden and Davies, 1975), but no previous attempt has been recorded to neutralize a rotavirus other than culture adapted calf rotavirus. It may be that field isolates of rotavirus that are not culture adapted cannot readily be completely neutralized, even by homologous sera. However, the failure of lamb rotavirus antiserum to neutralize calf rotavirus indicates the distinct nature of the lamb rotavirus.

The relatively short period of the primary antibody response of about 2 months may mean that CF and IF techniques are not satisfactory for screening

late convalescent sera for evidence of infection, unless reinfection occurs to increase antibody levels.

The epidemiology of rotavirus infections in calves suggests the possibility that latent infections may occur (Woode and Bridger, 1975). Although latent infection of rotavirus in the lambs was not confirmed, it is possible that either the methods used to provoke relapse were ineffective, or the techniques used to detect such a relapse were insufficiently sensitive.

SUMMARY

Inoculation of lamb rotavirus to gnotobiotic lambs produced a disease characterized by diarrhoea and anorexia. Rotavirus was excreted in the faeces of the lambs for several days. One-day-old lambs were more susceptible than 12-day-old lambs.

A serological response to infection was detected in all lambs by complement fixation and immunofluorescence techniques, using antigens prepared from calf rotavirus. Lamb rotavirus antiserum did not neutralize calf rotavirus, indicating the distinct nature of the lamb rotavirus.

No evidence of latent infections was detected by treating the lambs with an immunosuppressant four months after initial recovery.

ACKNOWLEDGMENTS

Miss E. Halliday and Mr J. Menzies provided excellent technical assistance.

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[*Received for publication, March 25th,* 1976]

Archives of Virology 55, 263—274 (1977)

Archives of Virology

Rotavirus Infection in Lambs: Pathogenesis and Pathology

By

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With 11 Figures

Accepted June 21, 1977

Summary

Experimental lamb rotavirus infections were studied by immunofluorescence, histopathology and electron microscopy of tissues from infected gnotobiotic lambs killed at intervals from the incubation period to recovery. The rotavirus was demonstrated by immunofluorescence only in epithelial cells of villi in the small and large intestine, and virus antigen was most abundant during the incubation period. An increased enterocyte turnover rate was suggested by the rapid movement of virus-infected cells to the villus tip, and this increase may be one of the basic pathogenic mechanisms of rotavirus infection. Principal histopathological changes were shortening of villi and sloughing of epithelial cells. These were greatest in the middle and posterior small intestine at the onset of diarrhoea, but regeneration occurred within a few hours. Virus morphology in tissues was similar to that reported in other species, and virus presence correlated well with histopathological change.

Introduction

The pathology of rotavirus infections has been studied in man (8), calves (10, 15), piglets (5) and mice (1). The techniques used have been immunofluorescence, histopathology, and electron microscopy, but only the observations in calves (10, 15) have utilised all 3 techniques. In all species examinations have been made only during a limited period in the course of the clinical disease.

Immunofluorescence studies demonstrated that rotaviruses infected the villous epithelial cells of the small intestine, and it was suggested that a wave of infection in these cells passed along the small intestine from the anterior end (10). Principal histopathological changes recorded were shortening of villi, loss of columnar cells from villi and their replacement with cuboidal cells (5, 10). Ultrastructural studies revealed damage to the microvilli of the villous epithelial cells and the presence of virus particles within cisternae of the rough endoplasmic reticulum (1, 5, 8, 15).

This paper describes the pathology of lamb rotavirus infections, studied by immunofluorescence, histopathology and electron microscopy from the incubation period to recovery.

Materials and Methods

Animals

Eight gnotobiotic lambs were infected orally when 2—4 days old with 2—3 ml of a bacteria-free 20 per cent faecal filtrate containing lamb rotavirus from the second, third, or fourth gnotobiotic lamb passage (13). One lamb was killed at each of the following hours after infection (p.i.): 12, 18, 27, 42, 48, 72, 96 and 144. Two gnotobiotic lambs were kept as uninfected controls; one was killed at 4 and the other at 6 days of age.

Necropsy Procedures

Lambs were deeply anaesthetised with sodium pentobarbitone. Segments were obtained from spiral colon, caecum and from 3 sites from small intestine: anterior, approximately 20 cm posterior to the pylorus; middle, approximately equidistant from the pylorus and the ileo-caecal junction; and posterior, approximately 20 cm anterior to the ileo-caecal junction. The lambs were then killed by exsanguination and gut contents collected. In addition, tissues were taken from the abomasal fold, kidney, liver, lung, mesenteric lymph node, myocardium and spleen.

Histological and Ultrastructural Methods

Segments of small intestine and some colon segments were fixed immediately by immersion in 1 per cent glutaraldehyde in phosphate buffer (pH 7.4). Slices of mucosa 1 mm thick were taken for electron microscopy (EM) and the remainder of the tissue transferred to 10 per cent buffered formal-saline for histology. All other tissues were collected directly into formal-saline. Additional portions of all tissues were frozen in a CO_2-isopentane freezing mixture prior to storage at —70° C for subsequent immunofluorescent (IF) examination.

Following post-fixation in a modified Bouin's fixative (190 parts sat. aq. picric acid, 10 parts 40 per cent formaldehyde, 5 parts glacial acetic acid) tissues were processed to paraffin-wax, and 5 μm sections cut and stained by Mayer's haemalum and eosin (HE). Other stains used were Pollak's trichrome and the periodic acid-Schiff (PAS) technique.

Selected areas of mucosa were post-fixed in osmium tetroxide and processed through graded alcohols for embedding in Araldite. Suitable areas for ultrathin sectioning were selected from Giemsa-stained 1 μm Araldite sections.

Immunofluorescence

Frozen tissues were mounted on microtome chucks and 6 μm sections cut on a cryostat. Tissues were stained with gnotobiotic lamb antiserum to lamb rotavirus, followed by fluorescein-conjugated rabbit anti-sheep globulin. Control sections were stained directly with the conjugated globulin.

Virus Isolation

Faecal samples collected daily and intestinal contents taken at necropsy were examined for rotavirus by immunofluorescence on cell cultures (14).

Bacteriology

Faecal swabs were taken at intervals during infection and at necropsy. No bacteria were isolated from the lambs killed 12, 18, 27, 42 and 48 hours p.i. *Staphylococcus epidermidis* was isolated from lambs killed at 72 and 96 hours p.i., a *Micrococcus* from the lamb killed at 144 hours p.i., and a *Bacillus* sp. from the two control lambs. No pathogenic significance is attached to any of these bacteria.

Results

Clinical and Virological Studies

The lamb killed 12 hours p.i. was clinically normal. The other seven infected lambs developed diarrhoea at 15—20 hours p.i., and two of these were also dull and showed abdominal discomfort. All infected lambs had diarrhoea until the

time of death except for the lamb killed 144 hours p.i. The control lambs were normal throughout the experimental period.

No rotavirus was detected in the faeces of the lambs before infection. Rotavirus was isolated from faeces of all diarrhoeic lambs and at necropsy from intestinal contents of all infected lambs except that killed 144 hours p.i. No virus was isolated from the uninfected control lambs.

Immunofluorescence

Specific IF staining was detected in the epithelial cells of the villi of the middle and posterior small intestine, and to a lesser extent in the anterior small intestine, caecum and colon (Table 1). Scattered cells in the lamina propria, presumably eosinophils, also fluoresced. The lamb killed 12 hours p.i. showed fluorescence in the epithelial cells covering the apical half or more of most villi in the middle and posterior small intestine (Fig. 1). By 18 hours p.i., fluorescence was observed in cells only over the tips of the villi (Figs. 2 and 3). Thereafter single fluorescent cells appeared sporadically on villi until 96 hours p.i. (Fig. 4). No specific fluorescence was detected in any tissue other than the intestine, nor in any tissue from the control lambs. IF staining was not evaluated in the spleen and mesenteric lymph node due to non-specific staining.

Table 1. *Immunofluorescent staining of lamb intestine for rotavirus antigen*

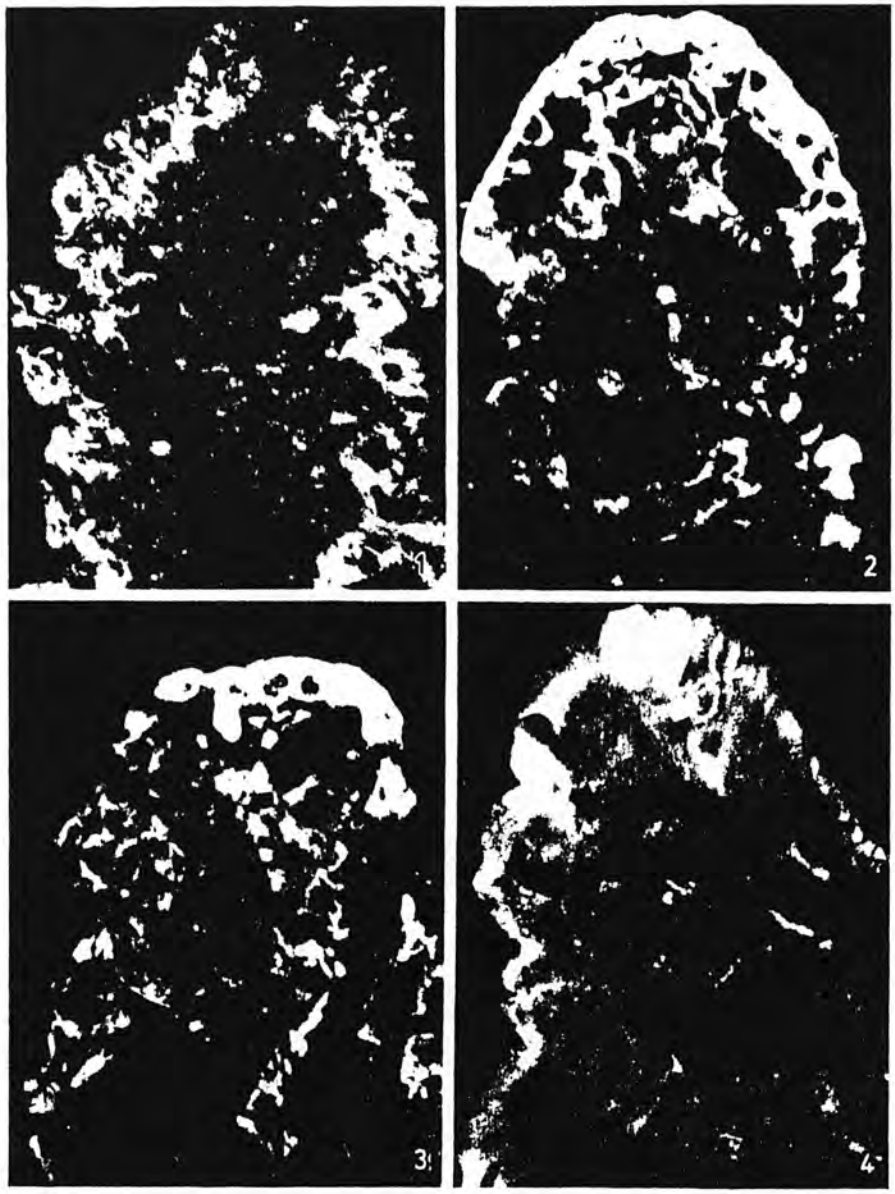
Time killed (hours p.i.)	Small intestine			Large intestine	
	Anterior	Middle	Posterior	Colon	Caecum
12	−	++++[a]	++++	−	−
18	++	+++[b]	+++	++	++
27	−	++[c]	++	+	++
42	+	+[d]	−	−	++
48	−	+	+	+	−
72	++	−	−	−	−
96	+	+	+	−	+
144	−	−	−	−	−
Control	−	−	−	−	−
Control	−	−	−	−	−

[a] ++++ Continuous fluorescent epithelial cells present over at least distal half of villi
[b] +++ Continuous fluorescent epithelial cells present over tip or distal third of villi
[c] ++ Sporadic fluorescent epithelial cells present in most villi
[d] + Sporadic fluorescent epithelial cells present in a few villi

Pathology

Focal congestion of the middle small intestine was found in the lamb killed at 12 hours p.i., while a more diffuse congestion of the small intestine and colon was present in the lamb which was scouring when killed at 18 hours p.i. At 12 hours p.i., the anterior lobes of both lungs were deep pink and partly consolidated. Similar areas were seen in the anterior lobes of both lungs of the lamb killed 72 hours p.i.

Histopathological lesions in small intestine at 12 hours p.i. were confined to middle and posterior sites, in which the villi were swollen and spatulate and the



Presence of viral antigen in tissues from lambs infected with rotaviruses, demonstrated by specific immunofluorescence:

Fig. 1. Jejunum, 12 hours p.i. (++++ Table 1)

Figs. 2 and 3. Jejunum, 18 hours p.i. (+++ Table 1)

Fig. 4. Caecum 27 hours p.i. (++ Table 1)

lamina propria was infiltrated by eosinophils and mononuclear cells. Normal intestinal columnar epithelium, similar to that of the control lamb (Fig. 5), was present at all three sites. At 18 hours p.i., changes at the anterior site consisted

Fig. 5. Villi from anterior ileum, control lamb. These are long, slender and covered by an epithelium of tall columnar cells. H & E × 100

Fig. 6. Villi from anterior ileum damaged by rotavirus infection, 18 hours p.i. These are short and spatulate, with deeply crenated surface epithelium. Sloughing of surface cells can be seen at villous tips. H & E × 100

only of plugging of submucosal capillaries by neutrophils. In middle and posterior sites, many villi were shortened and distended by an infiltrate of macrophages and small numbers of neutrophils. The epithelium over these villi was often markedly crenated, and some sloughing of cells at villus tips had usually occurred (Fig. 6). In some villi, the distal and lateral epithelial cells were either absent, or flattened and necrotic with deformed nuclei (Fig. 7). Capillaries in the mucosa and submucosa were dilated and plugged with neutrophils. The colonic and caecal mucosae contained petechial haemorrhages but were morphologically intact. Capillaries between the muscular coats, and those in the submucosa and subserosa, were plugged with neutrophils. At 27 hours p.i., the only change seen was shortening of villi at the posterior site, with an infiltration of eosinophils; thereafter pathological changes were not detected in any gut site.

In non-alimentary tissues, changes were not so marked. Numerous macrophages containing pleomorphic refractile eosinophile intracytoplasmic bodies were seen in the medullary sinuses of the mesenteric lymph node at 12, 18, 27 and 96 hours p.i. These bodies stained intensely by a trichrome method (Fig. 8), but

Fig. 7. Anterior ileum villus from the same lamb as in Figure 6, at higher magnification. The villus is swollen, its distal one-third denuded of surface cells, and surviving epithelium is either cuboidal or flattened. Pyknotic nuclei denote cells undergoing necrosis. Neutrophils can be seen in the lamina propria and local capillaries. H & E × 400

were PAS-negative. Focal accumulations of mononuclear cells were found in the liver at 12 and 144 hours p.i., and clusters of neutrophils were seen in liver sinusoids at most of the stages of the infection. Similarly, neutrophils were often seen in the splenic sinuses of infected lambs. An exudative bronchopneumonia was quite widespread in the lamb killed at 12 hours p.i.; the exudate was sero-cellular, both neutrophils and macrophages being found in alveolar spaces. A similar milder pneumonia was present at 72 hours p.i. No viruses were isolated from the lungs by standard tissue culture methods. No pathological changes were seen in other tissues. None of the changes described above were seen in the control lambs.

Fig. 8. Macrophages in medullary sinuses of mesenteric lymph node, infected lamb. Several cells (arrowed) contain multiple intracytoplasmic bodies of varying sizes. Trichrome stain × 1000

Electron Microscopy

Villous epithelial cells containing virus particles were extremely numerous at 12 and 18 hours p.i. at middle and posterior sites, but thereafter only isolated infected cells were found, up to but not beyond 96 hours p.i. At the anterior site infected cells were found only at 18 hours p.i. Infection of sub-epithelial macrophages was only seen at 12 and 18 hours p.i. Virus was never seen in crypt cells, goblet cells or colonic enterocytes. Infected cells contained large amounts of virus in distended cisternae of endoplasmic reticulum which usually formed large vesicles in the cytoplasm (Fig. 9). The virus particles consisted of central electron-dense cores (mean diam. 30 ± 3 nm) surrounded either by a zone of lesser density bounded by an electron-dense membrane (mean particle diam. 85 ± 4 nm) (Fig. 10), or more frequently by radiating filaments. Virus-associated lipid was frequently seen in the vesicles and cytoplasm. The vesicles were often closely associated with virus precursor material or viroplasm, and frequently with electron-dense material forming circular or paired ring structures of unknown derivation (Fig. 11). The presence of the latter often facilitated the identification of virus-infected cells at lower magnifications. Microvilli were usually undamaged

Fig. 9

in infected cells and resembled those of normal enterocytes. Although abbreviated or fused microvilli were found in some infected cells, these features were more usually associated with cells in which no virus particles were evident. At 12 and 18 hours p.i. only, infected cells were often seen in the process of separation and extrusion (Fig. 11), or were found sloughed off into the lumen of the gut.

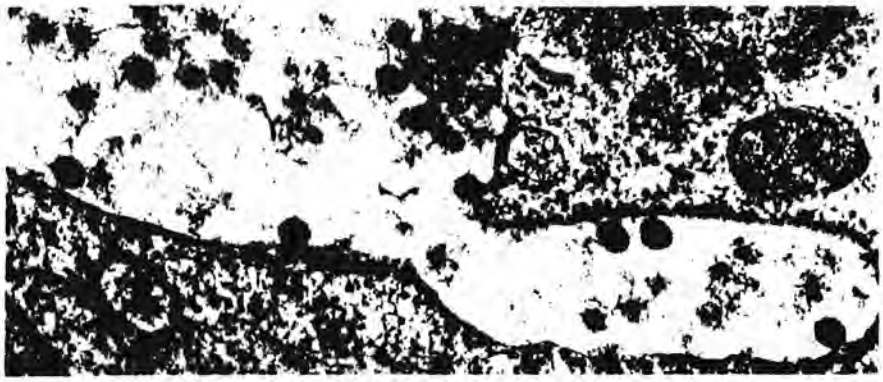


Fig. 10. Distended cisterna of endoplasmic reticulum containing virus particles with dense cores surrounded either by clearly-defined zones of lesser density, or by radiating filaments. × 60,000

Discussion

Lamb rotavirus multiplied mainly in villous epithelial cells of the middle and posterior small intestine, with smaller numbers of virus-containing epithelial cells present in anterior small intestine, caecum, and colon. The presence of virus identified by IF and EM correlated with the distribution of histopathological changes in the gut. The greatest amount of virus in villous enterocytes and maximum histopathological damage occurred during the incubation period and immediately after onset of clinical disease. All evidence of rotavirus infection had disappeared by 6 days p.i. Evidence of only slight rotavirus multiplication in anterior small intestine has been observed also in other species. Of 7 calves killed in the first 6 hours of rotavirus diarrhoea, only 3 showed evidence of virus multiplication at this site (10). Similarly, in pigs the upper small intestine was less severely affected than middle small intestine (5).

The histopathological changes seen in the small intestine, are similar to those in calves (10) but less severe than in piglets infected with calf rotavirus (5). Since immunofluorescence studies failed to demonstrate the presence of rotavirus anti-

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Fig. 9. Villous epithelial cell infected with rotaviruses, 18 hours p.i. The microvilli vary in length, and the terminal web is poorly developed. Cisternae of endoplasmic reticulum, often distended to form large vesicles (arrowed), enclose numerous virus particles, sometimes associated with lipid material (lip). Large and small masses of coarsely-granular viroplasm (v) can be seen in close relationship to these cisternae. For Figures 9—11 (electron micrographs) tissues were stained by lead citrate and uranyl acetate. Total magnification × 12,500

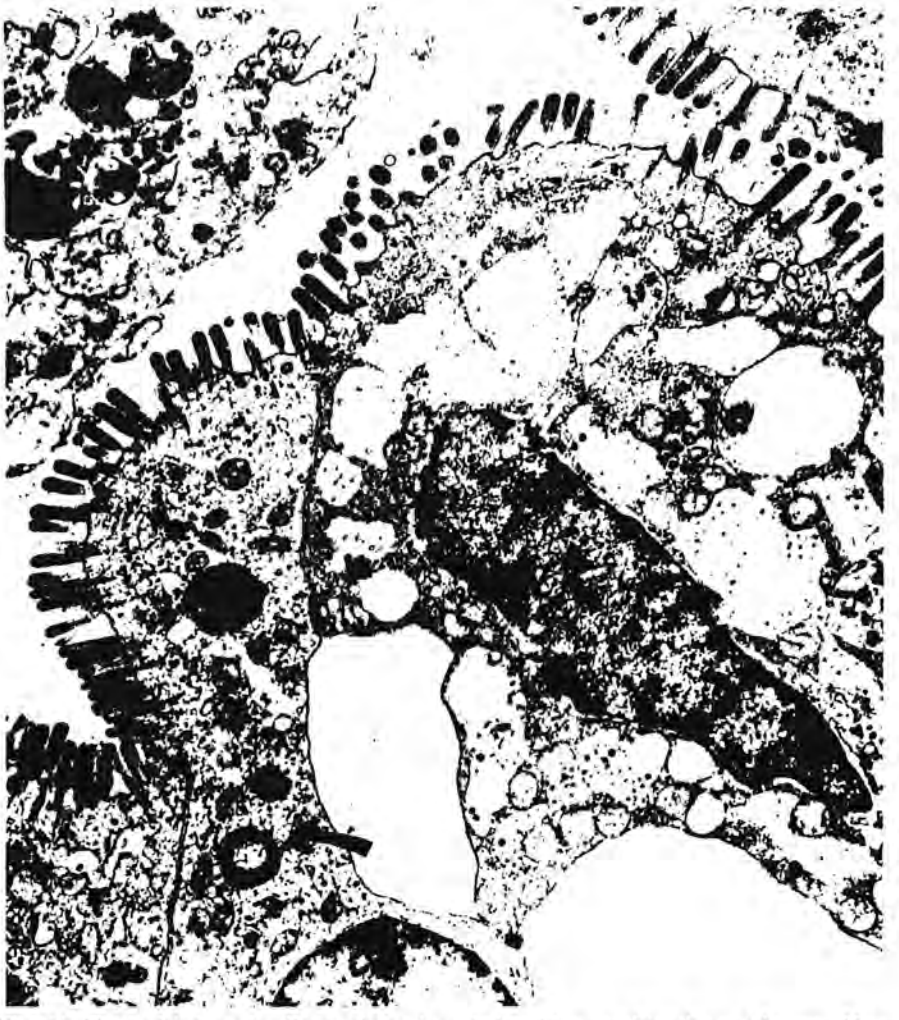

Fig. 11. Typical infected cells on the point of sloughing, and in the gut lumen. Note the pyknotic nucleus of the right-hand cell, and the large spaces between adjacent lateral plasmalemmata. A dense ring-shaped structure (arrowed), of unknown derivation but commonly seen in infected cells, is present in the left-hand cell. ×9000

gen in tissues other than the gut, it is not possible to relate the pulmonary and hepatic changes found in several infected lambs to rotavirus infection. The intracytoplasmic bodies seen in macrophages of the mesenteric lymph nodes may represent uptake of non-viral antigenic material which had passed from the gut lumen through the basal lamina following surface cell damage in the acute phase of virus infection.

The pathological changes and virus morphology detected by EM examination of villous epithelial cells were similar to those described for rotavirus infections in

other species (1, 5, 8, 15). Large (85 nm) and small (65 nm) virus particles were seen in infected human infants (8) and mice (1) and in a single experimental lamb (9). These may correspond to the 2 particle sizes described in negative contrast EM (4). In the present study only the larger size of complete virus particle was observed.

The electron-dense irregular or ring structures often found near virus-containing vesicles have apparently not been previously described. They were observed in addition to viroplasm and were in constant association with virus. Their significance is not known.

IF examination showed rotavirus-infected cells over the apical half or more of each villus during the incubation period, but only at the tips of the villi as infection proceeded. As infected cells migrate to the tip of each villus they may be replaced with noninfected cuboidal cells from the crypts. These newly-formed cells may be lacking in rotavirus receptors (7). However, the suggestion of a wave of infection progressing posteriorly from the anterior small intestine (10) has not been substantiated in this study.

The movement of virus-infected cells to the tips of the villi can be used as an indicator of cell replacement time, i.e. the time taken for epithelial cells in the small intestine to travel from the crypts to the tips of the villi. At 12 hours p.i., rotavirus-infected fluorescing cells were present on the distal half or more of each villus, but 15 hours later at 27 hours p.i. these infected cells had been mostly shed and replaced with non-fluorescing cells. A time of 15 hours for enterocytes to move along most of the villus length contrasts with a normal replacement time of at least 2—3 days in the neonatal lamb (11). Thus rotavirus infection resulted in a rapid replacement of damaged epithelial cells. Such an increase would result in the presence of immature cells on the villi, with a reduced absorption and digestive enzyme activity (12). This correlates well with the finding of reduced mucosal lactase in human infants and calves infected with rotavirus (2, 6). The evidence presented suggests that diarrhoea in rotavirus infection arises initially from epithelial cell destruction with subsequent rapid epithelial replacement leading to decreased absorption and impaired digestion. This results in the accumulation in the large intestine of lactose which ferments with consequent stimulation of fluid secretion (3).

Acknowledgments

We thank Mr. B. Mitchell and his staff for the supply and care of the gnotobiotic lambs.

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Received May 16, 1977

Printed in Austria

GASTROENTEROLOGY 76:477-481, 1979

Small Intestinal Morphology and Epithelial Cell Kinetics in Lamb Rotavirus Infections

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Morphologic changes in the small intestine of rotavirus-infected gnotobiotic lambs were investigated by measurement of villi and crypts in histologic sections of jejunum, midgut, and posterior ileum. In midgut, villus atrophy developed within 12 hr of infection and was apparent until 72 hr after infection. Crypt hypertrophy was evident from 42 hr after infection until the end of the observation period (6 days after infection). Changes in posterior ileum were similar in extent, but jejunal changes were much less marked. The relatively mild effect in the jejunum is in accord with reports from other species, and provides a basis for questioning the assumption that human rotavirus affects mainly the foregut.

Studies of epithelial cell kinetics were made on midgut using a microdissection and metaphase accumulation technique on sequential samples from anesthetized lambs. An increase in the cell production rate per crypt per hour from the overall control level of 5.8 was detected by 48 hr after infection. The maximum level of 21.2 was reached 8 days after infection, and this had returned to near normal by 15 days after infection. This large and sustained increase in crypt cell production probably underlies other previously described functional abnormalities.

Rotaviruses have been implicated as important causes of diarrhea in children and the young of several animal species.[1] Studies based on viral immunofluorescence, histology, and electron microscopy in calves, piglets, and lambs, have led to the concepts (a) that rotaviruses infect mature epithelial cells in the small intestine, and to a lesser extent in the caecum and colon; (b) that infected cells are sloughed, leading to partial villus atrophy; and (c) that the atrophic villi are rapidly reclad with relatively undifferentiated crypt cells, which mature over a few days and lead to healing of the lesion.[2-6] Reduced disaccharidase and increased thymidine kinase levels have been observed in rotavirus-infected small intestine,[7, 8] which supports the contention that immature enterocytes are present on the villi during rotavirus infection.

The basic assumption underlying this hypothesis, i.e., that there is substantially accelerated production of immature enterocytes from the crypts, has not been tested. In this paper, the authors have performed experiments on lambs infected with lamb rotavirus[5, 9]; these experiments investigated morphologic changes in small intestine, and kinetic studies on crypt cell production with a metaphase accumulation technique, from initial infection through to apparent recovery.

Materials and Methods

Animals

Twenty-two gnotobiotic lambs were delivered by hysterectomy and maintained in plastic isolators. Fourteen lambs were infected when 2-4 days old with 2-3 ml of a bacteria-free 20% fecal filtrate containing lamb rotavirus from the second to fifth gnotobiotic lamb passage.[5, 9] Eight lambs were kept as uninfected controls.

Experiment 1: Histologic Observations

One lamb was anesthetized with pentobarbitone sodium at each of the following hours after infection: 12, 18, 27, 42, 48, 72, 96, and 144 hr. Control lambs were anesthetized at 72, 96, 96, 144, and 144 hr of age, respectively. Segments of small intestine were collected from jejunum, midgut, and posterior ileum of anesthetized lambs into formol-saline as described[5] or into corrosive formalin.

Received August 7, 1978. Accepted October 11, 1978.

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The authors thank Mr. J. Menzies for skilled assistance and Mr. M. McLauchlan for help with the statistical analysis.

0016-5085/79/030477-05$02.00

Table 1. Histologic Measurements of Villi and Crypts (Experiment 1)

Time of sample	Villi			Crypts		
	Jejunum	Midgut	Posterior ileum	Jejunum	Midgut	Posterior ileum
hr postinfection		*(μm, mean ± SE)*			*(μm, mean ± SE)*	
Controls	600 ± 15	540 ± 17	614 ± 20	110 ± 5	100 ± 3	94 ± 2
12	527 ± 16	335 ± 21[a]	427 ± 19[a]	116 ± 7	94 ± 3	101 ± 4
18	552 ± 22	213 ± 5[a]	NS	116 ± 7	105 ± 3	NS
27	489 ± 24[b]	279 ± 10[a]	231 ± 8[a]	154 ± 4[a]	106 ± 7	111 ± 5[b]
42	541 ± 16	334 ± 23[a]	192 ± 9[a]	134 ± 6	124 ± 4[a]	102 ± 6
48	579 ± 33	307 ± 13[a]	278 ± 8[a]	153 ± 7[a]	125 ± 5[a]	143 ± 7[a]
72	606 ± 20	475 ± 20	321 ± 27[a]	223 ± 8[a]	134 ± 6[a]	116 ± 6[a]
96	531 ± 15[c]	681 ± 18[b]	346 ± 20[a]	152 ± 7[a]	151 ± 4[a]	104 ± 8
144	699 ± 33[b]	432 ± 8[c]	285 ± 13[a]	153 ± 6[a]	136 ± 6[a]	100 ± 5

Significance of deviation from control values. NS = no sample.
[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

Hemalum and eosin stained 5 μm sections were examined, and villus heights and crypt depths measured by ocular micrometer on 10 properly orientated villi and crypts at each site of small intestine.

Experiment 2: Microdissection and Epithelial Cell Kinetics

At each of 1, 2, 4, 8, 11, and 15 days postinfection an infected lamb and an age-matched uninfected control lamb were inoculated with vincristine sulfate (Oncovin, Eli Lilly & Co. Ltd) at 1 mg/kg by slow i.v. injection to block cells entering mitosis in metaphase. The animals were then anesthetized by inhalation of halothane and nitrous oxide and maintained anesthetized for 2 hr. At three time intervals over the 2-hr period, portions of midgut were ligated, and samples were placed in Clarke's fixative (75% absolute alcohol—25% acetic acid). After the last sampling, the lambs were killed.

The midgut tissues were stained by the Feulgen reaction. A line of villi with their associated crypts was cut from the edge of each specimen, and the lengths of 10 villi and 10 crypts were measured.[10,11] The crypts were separated from the lamina propria by pressure, and the number of cells in metaphase was then counted in each of 10 crypts per specimen.[11] The number of crypts per villus was calculated from counts of villus and crypt numbers on small areas of mucosa.[11]

The crypt cell production rate (CCPR—expressed as cells produced per crypt per hour) was calculated from a regression line of the accumulated numbers of metaphases on time after Oncovin inoculation. The rate of cell loss per villus per hour was calculated by multiplying the figure for CCPR by the mean number of crypts per villus for that section.

Results

Clinical and Virological Response

All infected lambs, except one killed 12 hr postinfection, developed watery diarrhea within 24 hr of inoculation, and most of them also showe apathy and decreased appetite. Control lambs continued to pass firm brown feces throughout the period of clinical reaction of the infected lambs.

No rotavirus was detected in the feces of lambs before infection or in the control lambs. Rotavirus was detected in feces of all diarrheic lambs, either by immunofluorescence on cell culture[12] or by enzyme-linked immunosorbent assay.[13]

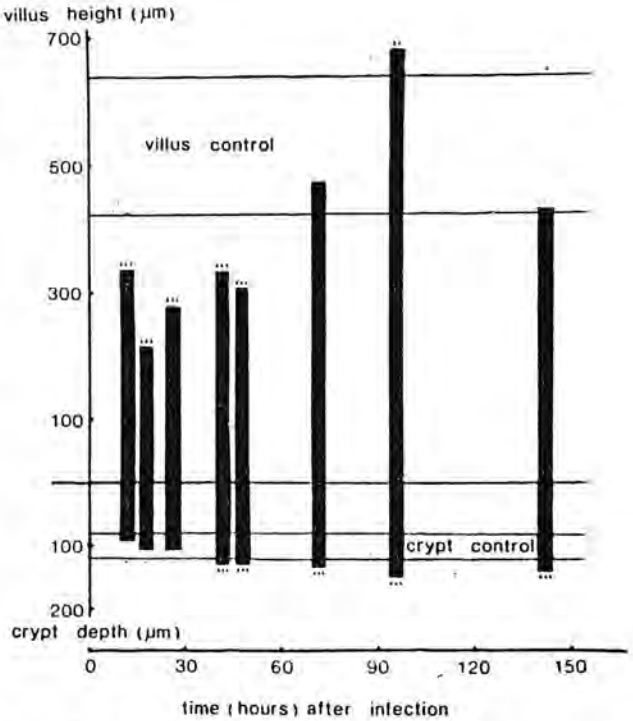

Figure 1. Villus height and crypt depth in midgut site (experiment 1). The crypt and villus controls are represented by a *band* for mean ± SD. The significances of the deviations from these control values are shown: —, not significant; X, $P < 0.05$; XX, $P < 0.01$; and XXX, $P < 0.001$.

Histology of Small Intestine

The villus heights and crypt depths at the three sampling sites in the five control lambs of 3–6 days of age (experiment 1) did not vary significantly with age. Normal measurements for villi and crypts at each site were therefore obtained by pooling observations for all five lambs. Measurements from individual infected lambs were compared with these normal values (Table 1).

The most marked changes were observed in midgut and posterior ileum. Significant villus atrophy occurred within 12 hr of inoculation and lasted for 2 days in midgut and throughout the experiment in posterior ileum. There was a temporary increase in villus height 4 days postinfection in midgut. By 1–2 days postinfection, the crypts showed a significant increase in length; this lasted throughout the experiment in midgut. In jejunum, a less consistent and less severe pattern developed, but villus atrophy and crypt hypertrophy were again evident.

The changes in midgut are illustrated graphically in Figure 1. As the abnormalities were more consistent and marked in midgut than in jejunum, and as it was sometimes difficult to select a posterior ileum sample free of Peyer's patches, it was decided to confine the microdissection and epithelial cell kinetic experiments (experiment 2) to the midgut site in the first instance.

Microdissection Measurements

The values obtained by measurement of villi and crypts in these specimens (experiment 2) were in proportion to but substantially greater than measurements in conventionally prepared sections from lambs in experiment 1, which is in accord with previous observations.[10] Villus atrophy was apparent in the lambs killed 1 and 2 days postinfection, while crypt hypertrophy developed by day 2 post infection and was still present at the end of the experiment.

Epithelial Cell Kinetics

All 12 lambs showed increasing accumulations of crypt cells in metaphase throughout the period of sampling. The regression lines of the numbers of accumulated metaphases with time are drawn in Figure 2.

The CCPR was calculated from the slope of the regression line. The CCPR for the control lambs did not vary significantly with age, and the production rate calculated from the combined regression line for all the controls was 5.8 cells per crypt per hour. The CCPR increased significantly by 2 days after rotavirus infection, reached a maximum of 21.2 cells/hr on the 8th day, and had returned to near normal values by the 15th day (Figure 3).

The number of crypts supplying cells to each villus varied between 2.1 and 4.2. The calculated cell loss per villus per hour increased roughly in proportion to the CCPR, to a maximum of 59.3 cells on the 8th day postinfection compared with a control rate of 15.5 cells/hr.

Discussion

The morphologic changes observed in the rotavirus-infected lambs confirmed and extended the authors' previous subjective descriptions.[5] Significant villus atrophy was apparent within 12 hr of infection and, in the distal site, was still present at 6 days postinfection. Crypt hypertrophy was first detected between 27 and 42 hr postinfection in all sites, and, in jejunum and midgut, was still present at 6 days postinfection. The measurements obtained and the sequence of events are in general agreement with those reported for pig rotavirus infections.[4, 6]

These measurements of villus height indicated that jejunum was less consistently and less severely damaged in lamb rotavirus infections than the other more distal sites. This observation corroborates the less severe jejunal changes found independently by histology, immunofluorescence, and electron microscopy.[5] The anterior small intestine has also been observed to be least damaged in pig rotavirus infections[4, 6] and in calf rotavirus infections in piglets.[3] The consistent occurrence of vomiting in infected children[14, 15] indicates damage to the proximal small

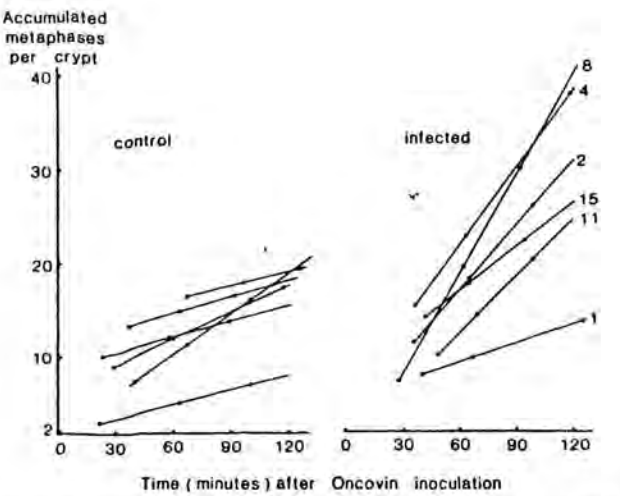

Figure 2. Accumulation of blocked metaphases in control and rotavirus-infected lambs. The regression *lines* are drawn for each lamb, and the *points* on each line represent the times when the lamb was sampled. The *numbers* beside the lines for the infected lambs indicate the day after rotavirus infection on which the lamb was killed.

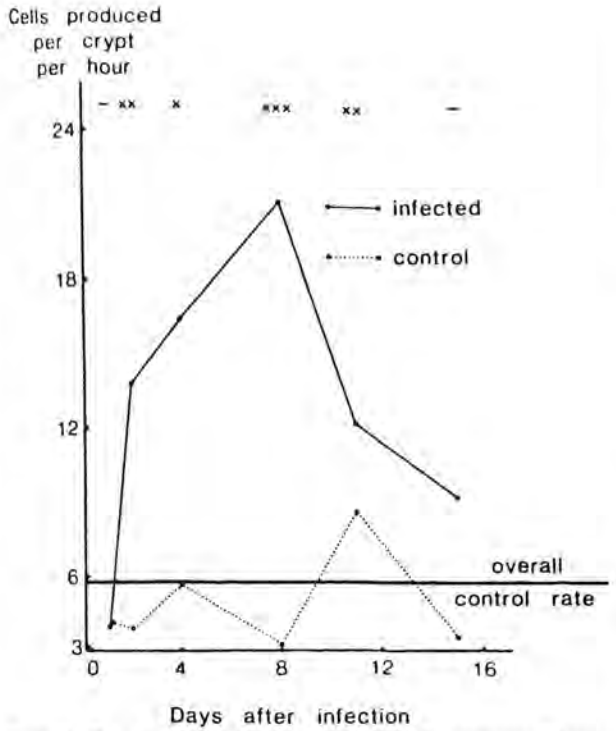

Figure 3. Crypt cell production rates (CCPR) compared for infected and control lambs. The data were converted to logarithms to minimize the positive association between means and variances. The regression lines for data from each infected lamb were compared with the regression line for all the control data after the differences between sheep had been removed. The levels of significance for the infected lamb CCPRs are shown: —, not significant; X, $P < 0.05$; XX, $P < 0.01$; and XXX, $P < 0.001$.

intestine, and studies in children are of necessity usually limited to this site.[7,16] Some workers using human rotavirus to infect piglets suggested that infection occurs primarily in the foregut,[8,17] whereas other studies on human rotavirus infections in monkeys[18] and calves[19] have reported involvement mainly of the mid and distal small intestine. Negative biopsy specimen results from children with suspect rotavirus gastroenteritis should be interpreted cautiously until this point is clarified.

By the end of the period of histologic observation (experiment 1), crypt hypertrophy in midgut was associated with normal villus height, and this suggests an increased turnover rate of epithelial cells. The cell kinetic studies of experiment 2 confirmed this, and show a substantial and prolonged increase in CCPR, to a maximum value at 8 days postinfection of approximately four times the normal level. This confirms previous suggestions that accelerated enterocyte migration might be the basic pathogenic mechanism underlying other abnormalities.[5,8]

The significance of this abnormality can only be partly assessed. Specific malfunctioning of glucose-coupled sodium transport,[9] impaired D-xylose absorption,[20] and reduced disaccharidase levels[7,8] have all been recorded in rotavirus infections and can probably be ascribed to increased CCPR, which leads to relatively immature undifferentiated enterocytes. It is likely that other functional abnormalities exist also as a result of the increased CCPR.

Diarrhea due to experimental rotavirus infections lasts for only a few days,[6,9] while the CCPR increased until 8 days postinfection and was abnormally high for nearly 2 wk. Thus, although the maximum CCPR is not responsible for the period of most severe diarrhea, it may cause a longer period of suboptimal gut function. Further work is necessary to determine the effect of rotavirus infections on such parameters as food conversion efficiency and liveweight gain.

This study leaves many questions unanswered, in particular as to what is the stimulus to prolonged increase in CCPR after villus atrophy has healed, and what are its functional effects. It does, however, confirm an important basic pathogenic mechanism of rotavirus infections.

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Reprinted from the Journal of the British Society of Gastroenterology— GUT
Volume 22, pages 114–119, 1981.

Lactose tolerance in lambs with rotavirus diarrhoea

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Gut, 1981, **22**, 114–119

Lactose tolerance in lambs with rotavirus diarrhoea

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SUMMARY It has been suggested that lactose malabsorption is an important factor in producing the diarrhoea of acute rotavirus infection. Accordingly, the lactose tolerance of gnotobiotic newborn lambs, infected with lamb rotavirus, has been investigated by clinical studies and tissue enzyme assays. Although lactase activity is low in affected areas of the small intestine, rotavirus infected lambs are not lactose intolerant as assessed by the measurement of reducing substances in the faeces, or by the clinical effects and blood glucose levels after a 5·8 mmol (2 g)/kg lactose load on the second day post-infection. Lactose intolerance could be demonstrated by using extremely high (29·2 mmol (10 g)/kg) doses of lactose, three or four times the normal dietary lactose intake. These experiments suggest that lactose-containing feeds (such as maternal milk) are not necessarily contraindicated in patients or animals with rotavirus diarrhoea.

Although gastrointestinal infection with rotavirus is an important cause of acute gastroenteritis in human children and in the young of many animal species, the pathogenesis of the diarrhoea has not yet been elucidated. Lactose malabsorption, with lactose intolerance and an osmotic diarrhoea, has been well documented in children with invasive bacterial gastroenteritis[1 2] and transient lactose intolerance is probably the commonest cause of diarrhoea with delayed recovery after acute gastroenteritis.[3 4] A possible role for lactose malabsorption in acute rotavirus diarrhoea has now been raised by the findings of lactase deficiency in duodenal biopsies from rotavirus infected children.[5 6] Disaccharidase deficiencies had previously been reported in small intestinal biopsies from adult volunteers infected with the Norwalk agent.[7 8] In a series of experiments in piglets infected with either rotavirus[9] or coronavirus (transmissible gastroenteritis virus (TGE))[10–13] Hamilton and his colleagues in Toronto have shown that during the diarrhoeal phase the enterocytes on the small intestinal villi migrate from the crypts at an accelerated rate and fail to differentiate fully. These immature, undifferentiated enterocytes are both disaccharidase deficient and deficient in Na^+K^+ATPase. This work provides the theoretical background to the postulates that sugar malabsorption[14 15] and/or defective sodium transport[16] are the primary mechanisms of diarrhoea.

Presence or absence of lactose malabsorption in acute rotavirus diarrhoea is of considerable practical importance in clinical management, not only in relation to recommendations as to whether or not disaccharides should be included in fluids for oral rehydration regimes[17] but also to allow for maintenance of nutrition by providing oral feeds which are not likely to be malabsorbed. For ethical as well as practical reasons this subject is difficult to investigate in human infants. However, a considerable amount of information relevant to the human disease has already accrued from work on the virology, pathology, and immunology of rotavirus infections in young domestic animals, and we are currently studying aspects of intestinal injury during and after rotavirus infection in newborn lambs. We have therefore extended our work to examine in detail a possible role of lactose intolerance in the acute diarrhoeal stage in this species. We have adapted a range of techniques which are used in human paediatric gastrointestinal practice to the investigation of these animals, and have supplemented *in vivo* methods by assay of lactase in tissues.

Methods

ANIMALS

Gnotobiotic lambs were delivered by hysterectomy and maintained in plastic isolators. Groups of lambs were infected when 2 days old with 2 ml of

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Received for publication 16 September 1980

bacteria-free 20% faecal filtrate containing lamb rotavirus from the fourth or fifth gnotobiotic lamb passage.[18 19] Other lambs were kept as uninfected controls. The animals were hand fed using sterile, evaporated cows' milk reconstituted with sterile distilled water. The lactose content of the reconstituted milk was 123 mmol/l (4·2 g/100 ml).

REDUCING SUBSTANCES IN FAECES

Specimens of faeces were diluted one in two with water and to 15 drops of diluted faeces a Clinitest tablet was added. The amount of reducing substances was estimated according to the colour resulting, ranging from 0%–2%.[20]

LACTOSE TOLERANCE TESTS

Lactose was dissolved in distilled water at a concentration of 292 mmol/l (10 g/100 ml), and sterilised by filtration (0·22 μm). Lambs were weighed within the isolator, and after an overnight fast lactose was administered orally in the animals given a dose of 5·8 mmol/kg and by stomach tube in the animals receiving 29·2 mmol/kg. Lambs were bled at 15 minute intervals for two hours after dosing. Heparinised plasma was separated and stored at −20°C and batches assayed for glucose by the method of Trinder.[21] Results were converted to values for whole blood by a correction factor using the packed cell volume.

LACTASE ASSAY

This was carried out in specimens of proximal jejunum, mid-small intestine, and terminal ileum from six infected animals one, two, four, eight, 11, and 15 days post-infection, and six uninfected age-matched controls. The animal was removed from the isolator, anaesthetised, laparotomy carried out, and the biopsies of three small intestinal sites obtained. Thereafter the animal was killed. Specimens were weighed, homogenised, and lactase was assayed by the method of Dahlqvist.[22] Results are expressed as units per gram of tissue wet weight.

Results

CLINICAL AND VIROLOGICAL

Examination of faeces confirmed the presence of rotavirus infection in all of the infected animals and in none of the controls. The clinical course was as previously described, with recovery by four days after infection.[18 19] Infected animals drank less milk than controls on the first and second days after infection, their mean daily intake being 650 and 880 ml compared with 900 and 1080 in uninfected age-matched controls. The difference between groups was significant, being $P<0·01$ on the day after infection. Thereafter infected and noninfected animals had milk intake from 1–1·3 l/day.

FAECES REDUCING SUBSTANCES

Samples of faeces were examined at intervals from two to 12 days after birth, in eight uninfected and nine infected animals. Faeces from four of the eight uninfected lambs contained reducing substances at up to 1% concentration as did faeces from seven of the nine infected animals. In both groups positive specimens were obtained from animals aged between 5 and 8 days. The diarrhoeal faeces from infected lambs did not contain reducing substances. The difference between these two groups is not significant (Fisher's test).[23]

LACTOSE TOLERANCE TESTS (5·8 mmol (2 g)/kg)

Lactose tolerance tests were carried out at 48 hours post-infection. Four uninfected and seven infected lambs were studied. There was no significant difference between the groups in blood glucose levels after the lactose load (Fig. 1). The animals remained clinically stable: in the infected lambs, there was no change in the character of the faeces, which were semiliquid due to rotavirus infection, and which did not contain reducing substances.

TISSUE LACTASE ACTIVITY

In the six uninfected control lambs there was no effect of age on the tissue lactase activity and so these six animals have been grouped and individual values from infected animals compared with the group, by

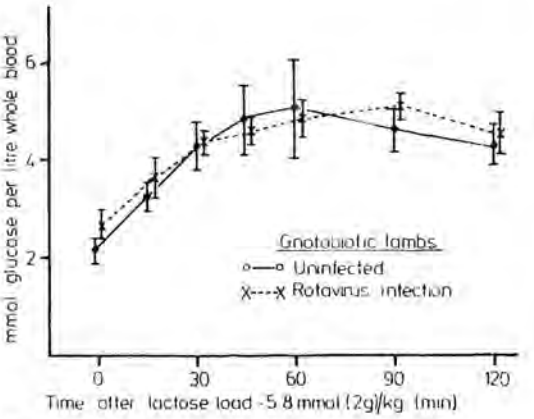

Fig. 1 *Blood glucose values (mean ± SE) after 5·8 mmol (2 g)/kg lactose load in groups of gnotobiotic lambs uninfected, or infected 48 hours previously with lamb rotavirus.*

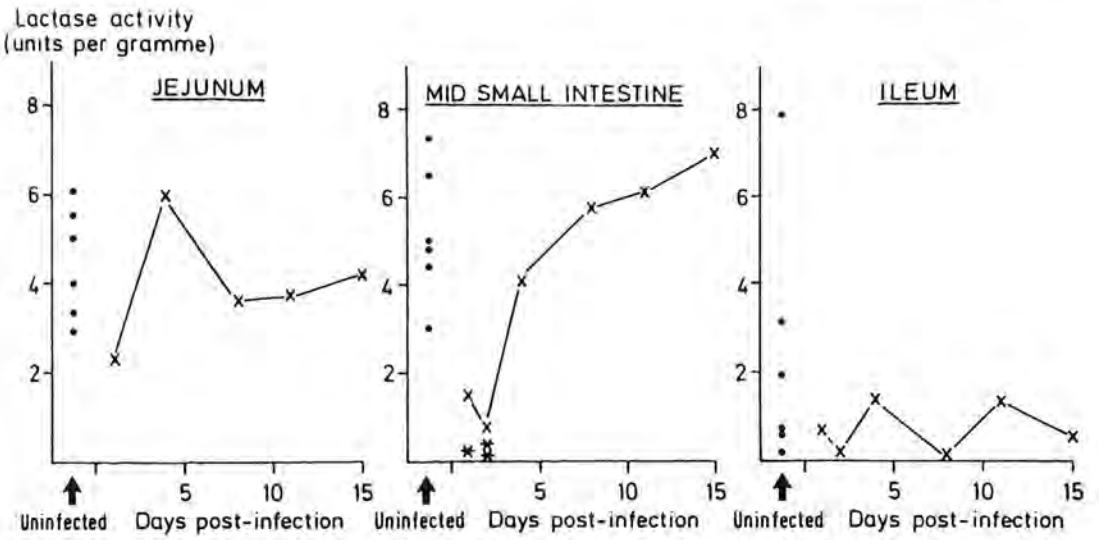

Fig. 2 *Lactase activity in small intestine of gnotobiotic lambs, uninfected, or one to 15 days after infection with lamb rotavirus infection (significance of difference from uninfected group* *$P < 0.05$ **$P < 0.01$).

using the Zm test.[23] The results are illustrated in Fig. 2. Lactase levels were abnormally low in the specimens taken from mid-intestine at one and two days post-infection, but had returned to normal values by the fourth day. Values in proximal and distal intestine were within the normal range.

LACTOSE TOLERANCE TESTS (29·2 mmol (10 g)/kg) In view of the apparent discrepancy between the findings of lactase deficiency in biopsy specimens, but apparently normal lactose tolerance by *in vivo* test, we proceeded to use a very large, totally unphysiological lactose load in an attempt to confirm that disaccharide intolerance could indeed be demonstrated in this experimental system. Lactose tolerance tests at 29·2 mmol/kg were carried out in two infected lambs at 48 hours post-infection and in two uninfected controls. Blood glucose levels are illustrated in Fig. 3. In the uninfected lambs the large dose of lactose produced a higher and more sustained rise in blood glucose than the 5·8 mmol/kg dose. In both of the infected lambs blood glucose also rose but the levels were lower than in the control animals. The control animals remained clinically well throughout the procedure, although their faeces were positive at ½% and 1% when tested for reducing substances. The two rotavirus infected lambs appeared well for some 90 minutes, but thereafter profuse watery diarrhoea which contained 2% reducing substances was superimposed on the rotavirus diarrhoea. This diarrhoea continued until the animals were killed some six hours later, at which time the ileum and colon were found to be grossly distended with watery yellow faeces.

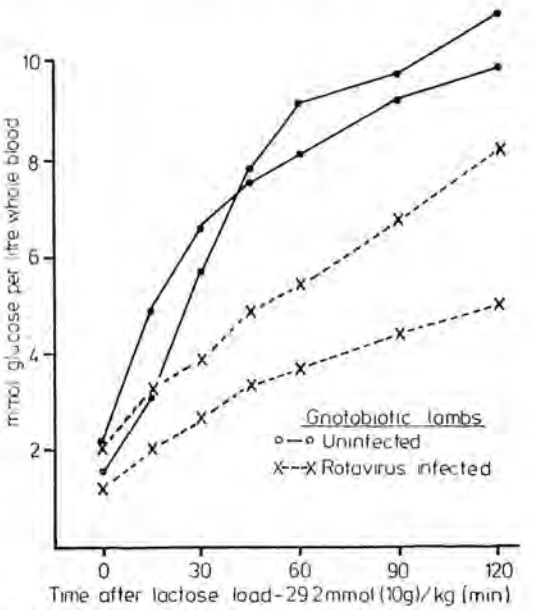

Fig. 3 *Blood glucose values after 29·2 mmol (10 g)/kg lactose load in two uninfected gnotobiotic lambs, and in two lambs infected 48 hours previously with lamb rotavirus.*

Discussion

Rotavirus infection of newborn gnotobiotic lambs is typical of the group of acute viral diarrhoeas in neonates. There is a brief acute illness with a low but significant mortality. Infected animals are lethargic and anorectic, diarrhoea lasts up to three or four days, and is accompanied by transient villous atrophy and crypt hyperplasia.[18 19 21] Clinical recovery, and histological recovery as assessed subjectively, are complete by four days post-infection, although measurements of epithelial cell kinetics have shown that crypt hyperplasia persists for at least two weeks.[21]

The Toronto group have been the main proponents of the theory that, from the second day after rotavirus or coronavirus infection, villi are clothed by immature enterocytes, the majority of which do not contain virus, and that the presence of these undifferentiated cells on the villi results in transient malabsorption and net water secretion—that is, diarrhoea. Among the evidence supporting their hypothesis and illustrating the differences between the viral diarrhoeas and the toxigenic bacterial diarrhoeas, are the findings of defective glucose stimulated sodium and chloride transport; low values for Na K ATPase; normal tissue levels of cyclic AMP; reduced sucrase activity and increased thymidine kinase activity in suspensions of enterocytes separated by a vibration technique from the villi.[9 10] Theil has reported similar results in rotavirus-infected piglets,[25] and we have recently reported that the basic assumption underlying this hypothesis, that there is substantially accelerated production of immature enterocytes from the crypts, is correct.[21] The overall crypt cell production rate in uninfected gnotobiotic lambs was 5·8 cells/crypt/hour, and values were found to be significantly increased above this level from day two post-infection, with a peak of 21·2 cells/crypt/hour on the eighth day—four days after apparent return to complete health in these lambs. This large and sustained increase in crypt cell production rate during and after rotavirus infection was surprising, for although an abnormality of cell kinetics with the production of immature undifferentiated enterocytes might explain the acute diarrhoea, cell kinetic changes have been even more profound after clinical recovery. Furthermore, our working hypothesis as to the reason for lactase deficiency was based on the suggestion by Rey and his colleagues some years ago,[26] that rapidly proliferating villus enterocytes will be deficient in lactase because of inadequate time for this disaccharidase to be synthesised by immature cells. Clearly, comparison of our results for lactase activity with the previously published cell kinetic measurements (which were performed in the same animals) show that changes in cell kinetics have not produced lactase deficiency, for tissue lactase levels were entirely normal in animals from the fourth day after infection.

Lactose malabsorption, if present, would contribute to the diarrhoea and illness of rotavirus infected animals and children by the production of an osmotic diarrhoea, by the loss of a substantial number of calories in the faeces, and possibly also by predisposing to hypoglycaemia. Since gnotobiotic lambs have no intestinal bacteria, measurement of faeces pH and of breath hydrogen excretion after a lactose load could not be used in the evaluation of lactose malabsorption in this system. However, we have used lactose tolerance tests, examination of faeces for reducing substances, and assays of tissue lactase levels to investigate this matter. Our results indicate that, although lactase levels are very low in the mid-intestine, where the viral induced damage is maximal, lactose intolerance does not appear to be present, at least at the doses of lactose which are taken by the animals spontaneously in their ingested milk or with tolerance tests using a dose of 5·2 mmol (2 g)/kg. Standard lactose tolerance tests had no adverse effects in infected lambs, and their blood glucose measurements showed normal absorption. Furthermore, there was no worsening of diarrhoea during or after the lactose load in infected animals. We have been able, by using a greatly increased lactose load, to produce severe watery diarrhoea in rotavirus infected animals but the character of the post-lactose diarrhoeal faeces was quite different from the faeces in rotavirus diarrhoea; and the amounts of lactose used were, in any event, three to four times more than would be ingested spontaneously either by an animal or by a human infant. In the healthy lambs, higher and more sustained levels of blood glucose after the 29·2 mmol (10 g)/kg lactose load, indicate that these animals have considerable reserve capacity for lactose absorption, when tested by a conventional lactose load, and when balanced against their normal milk intake. After rotavirus infection, the intestinal damage has caused a reduction in this reserve of lactase activity, though the animals are still tolerant of the amount of dietary lactose in milk.

It could be argued that lactose intolerance may not be present in those rotavirus infections which spare the jejunum, but will occur in rotavirus infection with a more proximal distribution—for example, in the calf.[27] However, this is not the case. In our studies of rotavirus infected calves (Snodgrass, unpublished) we have again failed to detect reducing substances in diarrhoeal faeces. The enzymes present in relatively undamaged areas of the small intestine

(jejunum in lamb rotavirus infection, ileum in calf rotavirus infection) appear to be adequate for absorption of dietary lactose in these species.

In human children, discrepancy between a state of lactose tolerance or intolerance, and the lactase content of a single jejunal biopsy has been recognised.[28] Again, the explanation is almost certainly that many enteropathies are patchy, and lactose is absorbed by the relatively undamaged parts of the intestine. It should be emphasised that, although pathological changes have been demonstrated in duodenal biopsies from rotavirus infected children, there is no information as to the relative severity of mucosal damage in proximal, mid and distal small bowel in humans. It is likely that some parts of the human small intestine are spared in rotavirus infection, for the reports of clinical aspects of acute rotavirus diarrhoea do not highlight malabsorption as a significant clinical problem.[29 31]

In children who are slow to recover after an acute gastroenteritis, lactose intolerance is not infrequently present. From our work it would seem that, as lactose intolerance is not a feature of the acute rotavirus diarrhoea, some mechanism other than a delayed recovery from rotavirus associated enteropathy should be sought to explain lactose intolerance. Milk intolerance in cows' milk protein hypersensitivity can itself be associated with lactose intolerance,[32] and hypersensitivity to cows' milk is regularly demonstrable in patients with the postenteritis syndrome.[33] This hypersensitivity mechanism producing disaccharide malabsorption deserves additional investigation, in animal models as well as in human clinical practice.

Although one must be cautious in extrapolating the results of experiments such as these to clinical practice in man, our findings suggest that, in the management of acute rotavirus diarrhoea, lactose-containing fluids (such as breast milk) are not necessarily contraindicated as agents for the maintenance of nutrition and hydration.

We acknowledge the skilled technical work of the staff of the Gnotobiotic Unit at the Moredun Research Institute; and are grateful to the staff of the Metabolic Unit, Western General Hospital, who carried out blood glucose measurements for this project. This work is supported by a grant from the Medical Research Council.

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Mixed infections in the intestinal tract.

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I. INTRODUCTION

Diarrhoea in young animals and children can be caused by any one of a variety of infectious micro-organisms acting alone. The outcome of such enteric infections in neonates is the result of complex interactions between host, infectious agent, and the environment. Superimposed on this complexity is the common observation that two or more enteropathogens may simultaneously infect the same individual with the possibility of an interactive effect. Enteropathogens may also interreact with other agents not regarded as primarily enteropathogenic in their own right.

II. OCCURRENCE OF COMBINED INFECTIONS

A.Calves.

Although many infectious agents have the potential to cause diarrhoea in young calves, only relatively few are known to do so commonly. Micro-organisms of wide distribution and proven enteropathogenicity are rotavirus, coronavirus, enterotoxigenic (K99+STa+) strains of E.coli (ETEC), Salmonella spp, and Cryptosporidium. (1) Discussion in this chapter will focus on the natural and experimental observations made on combined infections with these enteropathogens.

Few detailed epidemiological and microbiological surveys on diarrhoea in young calves have been performed, but there is general agreement that combined infections with more than one enteropathogen are common. (2,3) Surveys in the U.K. indicate that 15-20% of diarrhoeic calves and 2% of healthy calves

concurrently excrete more than one enteropathogen (4,5) (Table 1). An additional question as to whether primary infection with one agent leads to subsequent infection with another has not been investigated in the field.

The nature of combined infections varies with the prevalence of the different agents in a given region. In North America, combined infections of rotavirus and either enterotoxigenic E.coli or coronavirus are widespread.(2,3,6,7,8) This reflects the high prevalence of all three of these agents in the calf population. However in Europe, ETEC infection occurs as a clinically distinct and comparatively uncommon watery diarrhoea in very young calves of 1-2 days old only, and coronavirus infection also appears less common. (4,5,9,10) It is therefore unusual for ETEC infection to occur in combination with any other agent. On the other hand, combined infections of rotavirus with either coronavirus or more particularly with Cryptosporidium occurred in 4% and 11% of diarrhoeic calves respectively. (4) Coronavirus infections were observed to be 3 times more common in the presence of rotavirus infection. (5)

B.Piglets.

The range of common and proven enteropathogens in piglets contains a remarkably similar variety of viruses, bacteria, and protozoa to that occurring in calves - coronavirus (transmissible gastroenteritis (TGE) virus), rotavirus, ETEC (mainly K88+LT+), and Isospora suis. Reports on combined infections in suckling piglets with rotavirus and TGE virus (11), rotavirus and I.suis (12), and rotavirus with other enteric viruses (13,14), exist. However only Morin et al (15) have conducted a comprehensive survey of neonatal piglet diarrhoea for a range of infectious agents in a

number of individuals and outbreaks. In 78% of the piglets examined only one enteropathogen was detected, and the predominant agent involved was TGE virus which infected more than half of all piglets. Combined infections of any 2 of the 4 major enteropathogens listed above occurred in 12% of the piglets examined.

The syndrome of postweaning diarrhoea in piglets has been associated with ETEC infection and predisposing factors which may be either infectious, nutritional, immunological, or environmental. (16-18). In particular, Lecce et al (17) showed that ETEC infections were benign in weaned pigs unless they were concurrently infected with rotavirus, in which case typical postweaning diarrhoea occurred.

C.Children.

Many viral, bacterial, and protozoal infections can cause diarrhoea in young children. (19) However, reports of the occurrence of mixed infections in childhood diarrhoea are surprisingly scarce. In developing countries, hygienic considerations suggest that endemic infections must frequently coinfect children, as is often the case with young animals. The most comprehensive investigation of the occurrence of mixed intestinal infections in a developing country was carried out in Brazil by Guerrant et al. (19) They found that the commonest infections in children also were rotavirus and ETEC, but 14 of 35 children with enterotoxic coliforms were infected with other enteropathogens, and 12 of 24 children with rotavirus also simultaneously excreted other enteropathogens. Other reports of mixed infections from developing countries have been made from

Rwanda, where Cryptosporidium was diagnosed in combined infections with either Salmonella or Campylobacter in 2% of diarrhoeic children (20), and from Taiwan where Echeverria et al (21) observed that 7/11 ETEC infections were accompanied by rotavirus.

Concurrent intestinal infections with more than one enteropathogen are also common in industrialised western countries. In surveys conducted in the mid-1970s, infection with two or more viruses was detected in 14% of patients (22), and 13% of children with rotavirus were also found to be infected with other intestinal pathogens (23). However in a more recent large survey of children under 2 years of age hospitalised with acute diarrhoea, two or more intestinal pathogens were detected in 28% of patients (24). There was no difference in disease severity as measured by duration of diarrhoea and vomiting, occurrence of dehydration, character of stools, clinical and biochemical features, and general condition, between those who excreted single or multiple organisms (24).

C.Poultry.

Viruses are of considerable importance in enteritis of poultry. In a study of diarrhoeic turkey poults, rotaviruses, astroviruses, reoviruses, enteroviruses, and adenoviruses, were all detected, with combined infections occurring more commonly than single-agent infections (25).

III. EXPERIMENTAL STUDIES

The classical concept of primary viral infection predisposing to secondary bacterial infection has predominated in the design of experimental studies to investigate

interactions between neonatal enteric pathogens. Hence the infectious agents examined have been almost exclusively rotavirus and ETEC, and calves in particular have been used as experimental animals.

The first demonstration of interaction between rotavirus and ETEC was made by Gouet et al (26). They showed that in colostrum-deprived newborn calves a dose of 10^{10} ETEC produced a fatal diarrhoea, but that infection with either 10^{8} ETEC or rotavirus produced a nonfatal diarrhoea. However, when rotavirus infection at a few hours of age was followed by 10^{8} ETEC 24 hours later a severe fatal diarrhoea occurred. In calves a few days old, simultaneous infection with rotavirus and ETEC caused fatal diarrhoea, but no interaction was observed when ETEC infection was followed by rotavirus. Thus the basic concepts of the nature of rotavirus/ETEC interaction were determined : that interaction does occur, and that in particular rotavirus enables an ETEC infection to establish in circumstances where for reasons of age of animal or titre of inoculum it would not otherwise do so; and that this interaction can be most readily demonstrated when rotavirus is given prior to or simultaneous with the ETEC infection.

Subsequent studies have added to these observations by repeating and extending them, using calves of differing status from gnotobiotic to conventional suckling, using different ages of calves, and using different strains and doses of the infectious agents. In general, those experimenters who by reason of dose of inoculum or age of calf were able to produce a diarrhoea with ETEC infection alone did not show significant synergistic effects by superimposing rotavirus infection. For

example, Runnels et al (27) using 5-8 day-old gnotobiotic calves measured faecal dry weight, bacterial counts, viral shedding, and histological lesions. Combined infections tended to be slightly more severe than either rotavirus or ETEC infections separately, but they concluded that this effect was additive rather than synergistic.

On the other hand, those studies in which the ETEC infection alone was either abortive or subclinical, were often able to show that concurrent rotavirus infection produced a marked enhancement of the clinical disease. In experiments with calves over 1 week of age, Tzipori et al (28) were unable to produce diarrhoea with either rotavirus or ETEC alone, but combined infections caused clinical diarrhoea in either gnotobiotic or microbiologically conventional calves. Similarly Snodgrass et al (29) showed that ETEC were not able to colonise the intestine of 6 day-old conventional calves unless rotavirus was also present. Bacterial colonisation was shown to be greatly increased by rotavirus infection. The most detailed evidence for the mechanism of interaction was provided in experiments by Hess et al.(30) A small dose of 10^7 ETEC in specific pathogen-free calves under 24 hours old produced no clinical signs, and rotavirus alone caused a mild diarrhoea. A severe diarrhoea ensued when calves were infected with the two agents simultaneously or when the rotavirus was given prior to the ETEC. However if the calf was infected with ETEC before rotavirus, no exacerbation of disease was evident. With the combined infections, the incubation period was reduced, titres of both rotavirus and ETEC in the intestine were increased, and the severity of histological lesions was increased.

In experiments with postweaning diarrhoea in pigs, ETEC were shown to be unable to establish infection in 4-week old animals. However if the pigs were infected first with rotavirus the ETEC infection became established, and severe diarrhoea of long duration occurred. (17) This ability of rotavirus infection to enhance ETEC infection in postweaning pigs has been demonstrated by Tzipori et al also (16).

Experimental observations on rotavirus/ETEC interactions have also been made in other species. Increased mortality with combined infections has been observed in mice (31) and lambs, (32) and diarrhoea in foals could be caused by combined infections in situations where neither infection alone caused diarrhoea. (33)

There are few published records of experimental observations into combined infections other than those with rotavirus and ETEC. A synergistic action with both increased morbidity and mortality in precolostral calves infected with more than one of rotavirus, coronavirus, and BVD virus has been demonstrated (34). Tzipori et al (35) infected 2 lambs with rotavirus and Cryptosporidium, and did not show any exacerbation of the already severe cryptosporidial diarrhoea. Because of the common occurrence in calves of coinfections with rotavirus and either Cryptosporidium or coronavirus, further experimental investigations in this area are warranted.

IV. MECHANISMS OF INTERACTION.

There is a considerable amount of knowledge on the pathogenic mechanisms of the common enteric pathogens, but very little on the mechanisms of their interaction. Rotavirus and coronavirus multiply in the intestinal epithelial enterocytes.

Although they infect primarily the small intestine, the area infected is not consistent - some reports suggest jejunum is most severely affected,(36,37) while others found the lesions to be largely in the ileum. (38-40) Infection in the large intestine has also been noted occasionally. (38) Infected cells desquamate, producing villus atrophy and an epithelium either temporarily devoid of cells or lined with immature cuboidal cells produced from hyperplastic and hypertrophic crypts. These lesions produce defects in digestion, particularly of disaccharides, and impaired absorption. (41) There is also evidence that dysfunction in the intestine may continue for at least 2 weeks after rotaviral infection. (42)

Enteric bacteria have two well-recognised virulence mechanisms : enterotoxin production and invasion of mucosal cells. ETEC produce labile toxin (LT) and/or stable toxin (ST). LT attaches to enterocytes through specific membrane-bound gangliosides, and within the cytoplasm activates adenylate cyclase leading finally to active secretion of chloride. ST activates guanylate cyclase. (43) ETEC colonise principally the ileum, with lesser colonisation in the jejunum and none in the duodenum. (44) The most notable invasive bacteria are Shigella and Salmonella, although these organisms may also produce enterotoxin. Salmonella infect principally the ileum and large bowel.

Cryptosporidia invade the apical border of epithelial cells in ileum and to a lesser extent large intestine, causing widespread villus atrophy and fusion. (45)

Because of these widely differing pathogenic mechanisms, the potential exists for a variety of effects when two enteropathogens coinfect the same intestinal tract. For example,

it is conceivable that a jejunal rotavirus infection and an ileal cryptosporidial infection would have an additive pathophysiological effect with exacerbation of the clinical problem. Where both agents are infecting the epithelium in the same region of the intestine, these interactions are probably more complex and more specific.

Although there is clear experimental evidence that rotavirus enhances the ability of ETEC to infect the intestine, it is not known whether this is due to altered specific or nonspecific immune mechanisms or to enhanced cellular attachment. Bovine astrovirus has been shown to infect and damage the dome cell epithelium on intestinal Peyer's patches (46), which may result in abnormalities of immune function. It is not known what factors control the marked age restriction of ETEC infections in calves. It is possible that receptors present on a relatively mature epithelium at birth are important for bacterial attachment, but again it seems unlikely that the immature cuboidal epithelium produced after rotavirus infection would contain these same receptors. Altered bacterial adherence has been demonstrated after viral infection in the respiratory tract, with influenza virus increasing the adherence of several bacterial species to pharyngeal cells in vitro. (47) An alternative possibility is that rotavirus impairs nonspecific immune factors such as the mucus layer.

With these interactive mechanisms unknown, it is also possible that two enteropathogens could have mutually antagonistic effects, either through stimulation of nonspecific immune factors such as interferon, or through destruction or alteration of target cells. No such reactions have so far been reported or investigated.

V. CONCLUSIONS.

With many enteropathogenic infections endemic in the environment of the young animal or child, it is not surprising that mixed infections of the intestinal tract are frequently observed. This phenomenon has been reported much more commonly from veterinary medicine, and its significance in human medicine may well be underestimated. In most of the infections discussed, the presence of mixed infections is merely an added complication to the successful resolution of the case. However in postweaning diarrhoea of piglets the principal enteropathogen (ETEC) is not capable of initiating the disease on its own, but requires the presence of prediposing factors, one of which may be a concurrent rotavirus infection. In this case a combined infection is an essential prerequisite for disease to occur.

Experimental studies with combined infections have successfully confirmed the interactive effects of rotavirus and ETEC, but without ascertaining the underlying mechanisms. No experimental information is available on the potentially equally important virus-protozoa or virus-virus interactions exemplified by rotavirus infections combined with either Cryptosporidium or coronavirus, and these areas deserve a higher research priority.

It is also true that the microbial aetiology of diarrhoea in the young has not been fully elucidated, and that as information on newly-described enteropathogens becomes available new interactions will also be described, perhaps sometimes involving agents not normally recognised as enteropathogens in their own right.

Table 1. Detection of multiple infections (% incidence)

Survey	Calf status	No. of enteropathogens per calf#			
		0	1	2	3
Moredun*	healthy	65	33	2	0
	diarrhoea	29	56	15	< 1
Compton #	healthy	77	21	2	0
	diarrhoea	31	49	17	3

R * from Snodgrass et al, Vet. Rec., in press

from Reynolds et al, Vet. Rec., in press

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Veterinary Microbiology, 7 (1982) 51—60
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

INTERACTION OF ROTAVIRUS AND ENTEROTOXIGENIC *ESCHERICHIA COLI* IN CONVENTIONALLY-REARED DAIRY CALVES

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(Accepted 25 August 1981)

ABSTRACT

Snodgrass, D.R., Smith, M.L. and Krautil, F.L., 1982. Interaction of rotavirus and enterotoxigenic *Escherichia coli* in conventionally-reared dairy calves. *Vet. Microbiol.*, 7: 51—60.

A study was made of the effects of rotavirus and/or enterotoxigenic *Escherichia coli* (ETEC) on dairy calves born and suckled on the farm and subsequently reared in isolation. Calves were orally inoculated at 6 days old with either rotavirus (5), ETEC (7), rotavirus and ETEC (5) or remained uninoculated controls (4), and their reactions were recorded by clinical, microbiological, and pathological observations. Rotavirus infection consistently produced diarrhoea, while ETEC inoculated alone did not colonise the intestine. In dual infections, both rotavirus and ETEC multiplied, although the severity of diarrhoea was not greater than that caused by rotavirus alone. Some ETEC-inoculated calves developed subsequent naturally-acquired rotavirus infections, but in these no ETEC multiplication occurred. The results suggest that prior or simultaneous rotavirus infection is necessary to enable ETEC colonisation of the intestine in conventional calves of this age.

INTRODUCTION

Several enteropathogens, including rotavirus and enterotoxigenic *Escherichia coli* (ETEC) have been implicated commonly in the aetiology of neonatal calf diarrhoea (Acres et al., 1975; Morin et al., 1976; Moon et al., 1978; De Leeuw et al., 1980). ETEC infections occur most commonly in calves in which diarrhoea commences by 3 days of age (Acres et al., 1975; Moon et al., 1978) and experimentally it has been possible to produce diarrhoea, with ETEC, only in calves under 48 h old (Smith and Halls, 1967). On the other hand, rotavirus infections occur mainly in calves from 4 days to several weeks of age (Acres et al., 1975; Moon et al., 1978; de Leeuw et al., 1980). However, combined natural infections with rotavirus and ETEC do occur (Acres et al., 1975; Morin et al., 1976; Moon et al., 1978) although the significance of such

0378-1135/82/0000—0000/$02.75

combined infections is equivocal. Although most field studies have not detected any interaction (Acres et al., 1975; Moon et al., 1978), an increased severity of decrease has been suggested by Morin et al. (1976). Experiments in gnotobiotic calves have shown both a synergistic interaction producing a fatal disease (Gouet et al., 1978), and an interaction in which rotavirus aided ETEC colonisation but with little disease enhancement (Runnels et al., 1980). However, experiments with gnotobiotic animals exaggerate the ability of microorganisms to colonise the intestine, and the interaction of two organisms can more realistically be studied against the background interaction with a normal gut flora. Preliminary experiments by Tzipori et al. (1981) showed that rotavirus and ETEC inoculated together into conventional calves produced a more severe diarrhoea than that caused by either agent alone. The present study was therefore undertaken to investigate the interaction of rotavirus and ETEC in the production of diarrhoea in conventionally-reared calves aged 6 days.

MATERIALS AND METHODS

Animals

Jersey bull calves were collected when 1 to 2 days old from four dairy farms, where they had been kept from birth with their dams, in clean paddocks away from the main herd. Calves were brought to the laboratory where they were maintained individually in fibre glass tanks in isolation blocks. The calf accommodation was cleaned and fumigated with formaldehyde between experiments. Calves were fed 2 l of ultraheat-treated milk twice a day.

Twenty-one calves remained clinically normal, and were inoculated orally at 6 days of age. Five were inoculated with rotavirus, seven with ETEC, five received rotavirus and ETEC simultaneously and there were four uninoculated control calves.

Six calves were eliminated from the experiment before inoculation due to naturally-acquired diarrhoea and rotavirus infection and a seventh was eliminated due to detection of cryptosporidia although the calf remained clinically normal.

Inoculum

(1) *Rotavirus.* A gnotobiotic calf was inoculated orally with a 0.22-μm filtrate of intestinal contents from the fourth conventional calf passage of calf rotavirus field isolate C6 (Tzipori et al., 1980). A large volume of faeces collected from this calf 3 days after inoculation was homogenized with an equal volume of phosphate-buffered saline (PBS), dispensed in 4 ml aliquots and stored at −70°C. Rotavirus particles were readily visible by electron microscopic (EM) examination of unconcentrated preparations of this material. Each calf was inoculated with one 4 ml aliquot.

(2) *E. coli.* The enterotoxigenic calf strain B44 (09:K30—K99:NM) (Smith and Halls, 1967) which produces heat-stable but not heat-labile enterotoxin, was used. The organism was grown overnight on Minca-isovitalex agar (Guinee et al., 1977) and suspended in 0.15 M NaCl. The titre was estimated by optical density readings, and subsequent titration on sheep blood agar was used to determine the viable count. The calf inoculum consisted of a 10 ml suspension with a viable count of 1.2×10^9 to 1.3×10^{10} organisms per ml.

Observations

Faecal samples were collected from calves on at least two occasions prior to inoculation, and daily thereafter for 7 days or until the calf was killed. To determine severity of diarrhoea, faecal collection harnesses were fitted on ten calves, daily faecal output was weighed and the faeces dried to constant weight for dry matter estimation. All calves were examined at least once a day, particular attention being paid to the nature of the faeces. Serum was obtained from calves prior to inoculation.

From fourteen calves under terminal anaesthesia, five segments of intestine were taken: jejunum, approximately 50 cm distal to the duodenojejunal flexure; midgut; ileum, approximately 1 metre proximal to the ileocaecal junction, where possible in an area free of Peyer's patches; caecum; and spiral colon. An opened portion of gut was fixed flat in 10% formol-saline and processed for histological examination. Mean villus height and crypt depth at each small intestinal site were determined by measuring up to ten well-orientated villi and crypts using an ocular micrometer. A 5 cm length of small intestine including contents, or contents alone in the case of large intestine, was taken for enumeration and identification of *E. coli.* A 3 cm length of gut was filled with embedding medium (Lab-Tek Products) and frozen in a dry ice-isopentane sludge for cryostat sections. Immunofluorescence was used to detect K99 or rotavirus antigen in the sections.

Neutralization Test. Serum samples were tested for the presence of neutralizing antibody to tissue culture adapted calf rotavirus (McNulty et al., 1976) on MA 104 cells grown in microtitre plates. Titres are expressed as the reciprocal of the highest dilution giving complete neutralization.

Faecal Examination. Faecal samples were examined for rotavirus by enzyme-linked-immunosorbent assay (ELISA), using a technique similar to that described by Fahey et al. (1981), except that the conjugated enzyme was horseradish peroxidase rather than alkaline phosphatase. In addition, at least one faecal sample from all scouring calves was examined by EM to detect rotavirus and other enteric viruses.

Faecal swabs were cultured on both sheep blood agar and MacConkey agar overnight. Ten colonies of *E. coli* were randomly selected from the plates and subcultured on to Minca-isovitalex agar overnight. These *E. coli* were tested

TABLE I

Occurrence of diarrhoea and infection in experimental calves

Calf Group and number		Incubation period to diarrhoea (day p.i.[a])	Preparent period to rotavirus excretion (day p.i.)	Preparent period to B44 excretion (day p.i.)	Max B44 in faeces (no. detected of 10 colonies examined)	Day of necropsy (day p.i. or age for controls)	Sites of immunofluorescence		*E. coli* Titre in ileum (log_{10} viable count/5 cm)
							Rotavirus	K99	
Control	1	N[b]	—[c]	—	0	7	—	—	3.9
	2	N	—	—	0	7	—	—	5.1
	3	N	—	—	0	6	—	—	6.2
	4	N	—	—	0	8	—	—	5.2
Rotavirus	5	4	3	—	0	...[d]	...	...	...
	6	3	4	—	0	5	1,2	—	4.3
	7	2	4	—	0	...	...	...	...
	8	2	4	—	0	...	...	...	...
	9	2	3	—	0	...	...	...	...
ETEC	10	N	—	—	0	3	—	—	3.9
	11	N	—	1	10	...	...	...	...
+naturally	12	2	4	—	0	4	1,2,3	—	5.5
acquired	13	1	1	—	0	3	1	—	3.1
rotavirus	14	4	4	—	0	6	1,2,3	—	4.9
infection	15	4	6	—	0	...	...	...	...
	16	8	6	—	0	...	...	...	...
Rotavirus	17	3	3	2	10	4	1,2,3,4	—	8.7
+ ETEC	18	2	—	2	6	2	—	—	7.1
	19	3	3	2	8	7	—	—	6.0
	20	2	—	1	10	3	1,2	2,3	9.0
	21	2	4	2	10	6	—	—	3.3

[a] p.i., post inoculation.
[b] —, not detected.
[c] N, remained normal.
[d] . . ., not examined.
Sites of immunofluorescence: 1, proximal jejunum, 2, midgut, 3, distal ileum, 4, caecum.

for the presence of K99 pilus antigen by slide agglutination with a K99 antiserum prepared in rabbits by inoculation of a K99+ mutant of a K12 strain, with subsequent absorption of the serum by the parent K12 strain (Moon et al., 1977). Each of the ten selected colonies were also subcultured onto tryptose soya agar, autoclaved for 2.5 h at 121°C, and tested for 09 antigen by a tube agglutination test using 09 antiserum prepared in rabbits (Edwards and Ewing, 1972). *E. coli* that possessed both K99 and 09 antigens were considered likely to be B44.

RESULTS

Occurrence of diarrhoea

Calves had preinoculation serum antibody titres to rotavirus of 160-1280.

The four control calves continued to pass faeces of normal colour and consistency, remained alert and had a normal appetite.

All five calves inoculated with rotavirus developed diarrhoea after 2—4 days (Table I). The mean maximum daily faecal output increased from 45 ± 8 g to 1042 ± 78 g, and dry matter decreased from 28 ± 1% to 8 ± 1% (mean ± s.e.) (Table II). Two of these calves also became dull and anorectic.

TABLE II

Maximum daily output and minimum dry matter of faeces from diarrhoeic and normal calves (Mean ± s.e.).

Group	Maximum faecal weight/24 h (g)	Minimum faecal dry matter (%)
Normal calves	45 ± 8	28 ± 1
Rotavirus	1042 ± 78	8 ± 1
Rotavirus + ETEC	1072 ± 43	9 ± 2
ETEC (+ natural rotavirus infection)	990 ± 290	8 ± 3

Only two calves inoculated with ETEC did not acquire natural rotavirus infection (Table I) and both remained normal throughout. The other five calves in this group became diarrhoeic, and were subsequently shown to be infected with rotavirus. One of them exhibited partial anorexia.

The five calves inoculated with both rotavirus and ETEC all started to scour after 2—3 days and became dull and anorectic (Table I).

The severity of diarrhoea was similar in calves infected with rotavirus, or rotavirus and ETEC, or in calves with rotavirus infections acquired naturally (Table II).

Virological Examination

All calves inoculated with rotavirus, and four of the five dually-inoculated calves, became infected with rotavirus (Table I). In spite of the isolation precautions, five of the seven ETEC-inoculated calves acquired rotavirus infections which were detected both by examination of faeces and by immunofluorescent staining of gut sections. Although all small intestinal sites sampled and caecum could be shown to be infected with rotavirus on occasion, the most consistently infected part of the gut was the anterior jejunum. Rotavirus infections were not detected in other calves. Coronaviruses or other enteric viruses were not detected by EM examination.

Bacteriological Examination

B44 were considered to have colonised the small intestine if immunofluorescence with K99 antiserum was demonstrated in gut sections, and if adherent bacteria could be observed histologically. In addition, it was considered likely that isolation of 10/10 K99+ and 09+ colonies in faeces indicated multiplication of B44 to high titre in small intestine.

None of the control calves, nor any of those inoculated with rotavirus, excreted B44. In contrast all five animals inoculated with rotavirus and ETEC became infected with B44. Of the seven calves inoculated with ETEC alone including those with naturally-acquired rotavirus infections, only one (# 20) showed evidence of infection with B44 (Table I). This animal, which was killed while K99+ bacteria were present on epithelial cells in ileum, had the highest titre of bacteria in ileum.

The titre (mean ± s.e.) $\log_{10}$ viable count/5 cm of *E. coli* in ileum of control calves was 5.1 ± 0.5 (range 3.9 to 6.2) (Table I). Titres in jejunum and midgut were also within this range. Viable bacterial counts in all three small intestinal sites of all calves inoculated separately with either rotavirus or ETEC were within this normal range. In the dually-inoculated calves killed 2 to 4 days after inoculation, ileal *E. coli* titres of 8.3 ± 0.6 were significantly greater than titres in control calves ($P < 0.01$).

Histological Examination

The small intestine of control calves had long slender villi with intact columnar epithelium. The length of villi decreased from proximal to distal end of small intestine (Table III). No significant lesions were observed in calves in this group.

There was evidence of crypt hypertrophy and a patchy villus atrophy in calf # 10, killed three days after ETEC inoculation (Table III). No other lesions were detected.

Lesions in the rotavirus-inoculated calf and in the calves with naturally-acquired rotavirus infections were similar. Villi at all small intestinal sites

TABLE III

Measurement of villus height and crypt depth in small intestine of control and infected calves (mean ± s.e.)

Group	Villus height (μm)			Crypt depth (μm)		
	Proximal jejunum	Midgut	Distal ileum	Proximal jejunum	Midgut	Distal ileum
Control (4)	972 ± 46	909 ± 41	858 ± 34	269 ± 6	235 ± 9	245 ± 7
Rotavirus (1)	497 ± 53*	543 ± 29*	389 ± 28*	501 ± 26*	554 ± 18*	447 ± 16*
Rotavirus + ETEC (5)	434 ± 14*	503 ± 26*	529 ± 38*	317 ± 9*	374 ± 14*	332 ± 8*
ETEC + naturally acquired rotavirus (3)	510 ± 32*	605 ± 30*	425 ± 16*	426 ± 13*	447 ± 8*	337 ± 13*
ETEC (1)	912 ± 75	487 ± 24*	872 ± 42	332 ± 15*	418 ± 25*	404 ± 32*

*Differ significantly ($P < 0.01$) from control measurements.

sampled were short and broad (Table III). Epithelial cells varied from columnar through low columnar to cuboidal, with most abnormal cells present near the tips of villi, particularly in midgut and ileum. Occasional fusion of villi was noted in only one animal. The lamina propria was usually infiltrated with mononuclear cells and polymorphonuclear leucocytes. Crypts were significantly lengthened (Table III) and often contained many mitotic figures.

In the five calves with rotavirus and ETEC infection, villi were blunted, shortened and broadened to an extent similar to the rotavirus-infected calves (Table III). Epithelial cells were cuboidal or even squamous near the tips of villi. Fusion of villi occurred in three calves, particularly in midgut and ileum. In calf # 20, bacteria were seen adhering to epithelial cells, and tongues of epithelial cell layers protruded from the tips of many villi. The lamina propria was infiltrated with neutrophilic and eosinophilic polymorphonuclear leucocytes, and less abundantly with plasma cells, lymphocytes and macrophages. Crypts were hypertrophic (Table III) with many mitotic figures.

DISCUSSION

The major problem in using conventionally-reared calves proved, as anticipated, to be the occurrence of natural infections with enteropathogens in some calves. Experimental conditions were standardized to the greatest extent possible compatible with the inevitable variation present among conventional calves, and the microbiological status of all calves was carefully monitored. While the results must be treated with caution, they relate to the field disease in that they were obtained from conventional calves of an age in which, in our experience, diarrhoea occurs commonly.

Calves inoculated with rotavirus developed diarrhoea of moderate severity, and two of the five calves had reduced appetite. Histological lesions were similar to those described by Mebus et al. (1971). The inoculum of unfiltered

gnotobiotic calf intestinal contents containing an Australian rotavirus isolate was prepared in a similar manner to that used successfully to produce diarrhoea in calves with a rotavirus isolate from the U.K. (Snodgrass et al., 1980). The pathogenicity of these two strains in conventionally-reared calves suggest that use of a high titre inoculum may be important, and that previous failure to demonstrate substantial pathogenicity may have been due to use of lower titre filtered inoculum (Logan et al., 1979).

Of the seven calves inoculated with ETEC alone, only one (#11) showed any evidence of multiplication of the B44 strain inoculated. This is consistent with observations of others that only when given within the first 48 h of life can ETEC infection produce diarrhoea (Smith and Halls, 1967).

The five dually-inoculated calves all developed diarrhoea, all became infected with B44, and all (except #18) became infected with rotavirus. The consistent multiplication of B44 in this group was demonstrated by its excretion in faeces and by higher ileal titres. Thus simultaneous inoculation of rotavirus with ETEC enabled ETEC to multiply in all five calves while ETEC in the absence of simultaneous rotavirus inoculation colonized only one of seven calves.

This combined infection did not produce any increase in severity of disease, as measured by weight and dry matter of diarrhoeic faeces and extent of villus atrophy compared to that caused by rotavirus infection alone. However, dual infection was associated with loss of appetite in all calves, and villus fusion, which may be a feature of ETEC infection, was common (Pearson et al., 1978).

Diarrhoea occurred in five calves inoculated with ETEC, and in all these animals rotavirus infection was demonstrated. The naturally-acquired rotavirus infections were presumably responsible for the diarrhoea in these calves, as no ETEC multiplication was demonstrated. The severity of diarrhoea, the bacterial titres in the ileum and the histological findings were all similar to those of the group inoculated with rotavirus, and these calves should properly be considered with the rotavirus-inoculated group. The absence of ETEC multiplication in these calves in spite of rotavirus infection suggests that the timing of each infection may be important. In the naturally-acquired infections, rotavirus was generally detected later than in the calves inoculated with rotavirus. The establishment of ETEC infection in calves of this age may therefore require an initial or at latest a simultaneous rotavirus infection.

Other studies on combined rotavirus and ETEC infections have utilized gnotobiotic calves (Gouet et al., 1978; Runnels et al., 1980). In spite of the difference in the experimental systems, similar conclusions can be drawn. Runnels et al. (1980) demonstrated increased ileal colonisation by ETEC in the presence of rotavirus infection, and Gouet et al. (1978) showed that rotavirus infection can make a sublethal dose of ETEC become lethal. Thus in gnotobiotic calves ETEC readily colonise the intestine, and rotavirus infections enhance this colonisation. However, in conventional calves in this ex-

periment ETEC colonisation occurred in the presence of rotavirus infection while ETEC were eliminated in the absence of rotavirus infection. This supports the observations made by Tzipori et al. (1981). Thus in this study the interaction of rotavirus and ETEC in the intestine may be classed as synergistic rather than additive.

ACKNOWLEDGEMENTS

The authors wish to thank Jill Billington, Alan Harbinson, David Whittle and Karen Wilson for their technical assistance.

This work was supported in part by the Australian Meat Research Committee.

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Archives of Virology 52, 201–205 (1976)

Rotavirus Infection in Lambs: Studies on Passive Protection

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Accepted July 15, 1976

Summary

Gnotobiotic lambs were protected against rotavirus infection by the presence in the gut at the time of infection of colostrum or serum containing antibodies to rotavirus. This protection was observed even when passively-acquired antibody was not present in the serum of the infected lamb. Infection under these conditions may have conferred immunity to subsequent challenge.

Introduction

Rotaviruses have been recognised as a cause of gastroenteritis in neonates of several species including man, but immunological mechanisms of defence against rotavirus infectious have not been fully investigated. Two methods of protection have been used in calves. These were the use of a live tissue culture attenuated vaccine given at birth which produces protection within two days (6) and stimulation of the immunity of cows by the inoculation of inactivated virus in an attempt to confer passive protection to the calves through colostrum (6). Some success has been achieved using both these methods but the protective mechanisms have not been investigated.

The role of passively-acquired serum antibody is uncertain as both field and experimental observations (6, 12) indicate that such antibody in calves does not protect. However close scrutiny of a natural outbreak demonstrated a direct relationship between passively-acquired serum immunoglobulin in calves and resistance to rotavirus infection (5).

Rotavirus infections occur naturally in lambs (9), and disease is produced by experimental infection (8).

This study was designed to see if colostrum and serum containing antibody to rotavirus could protect neonatal lambs against experimental lamb rotavirus infection.

enteritis of pigs caused by a coronavirus (Haelterman 1965).

McNulty and others (1976a) suggested that in rotavirus infections the protective role of colostrum might be quantitative, and that ingestion of sufficient colostrum to produce circulating immunoglobulin levels of over 30 mg per ml might be protective. In this paper we describe experiments to investigate the respective roles of colostrum and serum antibody in protecting lambs against diarrhoea caused by rotavirus.

bacteriologically sterile. Serum preparations used were: Batch 1, pooled sera from normal sheep on two farms on which most sheep had serum antibody to rotavirus (NT 10, IgG 50·0 mg per ml); batch 2, serum from two, three month old, gnotobiotic lambs with a controlled rumen microflora (NT < 2·5, IgG 7·3 mg per ml); batch 3, serum from two sheep immunised by repeated intramuscular inoculations of lamb rotavirus (NT 3200, IgG 84·7 mg per ml). The three batches of serum were sterilised by filtration through 0·22 µm membrane filters.

Materials and Methods

Animals

Eight gnotobiotic lambs were used, 2 in each of 4 isolators.

Virus

A 20 per cent bacteria-free filtrate of intestinal content from the second gnotobiotic lamb passage of lamb rotavirus was prepared and given orally in 3 ml amounts. The titre of this filtrate was $10^{3.5}$ $TCID_{50}$/0.1 ml.

Virus Detection

The faeces of the experimental lambs were examined by homogenising a 20 per cent suspension in distilled water, clarifying at 1300 × *g* for 10 minutes, and filtering through a 0.45 μm membrane filter (Millipore). The resulting suspension was inoculated into maintenance medium on secondary calf kidney (CK) cells (8) grown on coverslips in tubes, and incubated at 37° C. After one day the coverslips were fixed in acetone, stained with rabbit antiserum to lamb rotavirus (8) and sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate (Wellcome), and examined for the presence of fluorescing cells.

Estimation of Immunoglobulin

The single radial immunodiffusion method (2) was employed, using standard ovine IgG and specific antiserum prepared as described previously (7).

Colostrum and Serum

Sera and first-day colostrum were collected from Dorset Horn ewes on a farm where the sheep were known to have a high incidence of antibody to rotavirus. The sera and colostrum samples were individually tested for rotavirus antibody by the indirect immunofluorescence (IF) test (8) and those samples positive at a dilution of $^1/_{40}$ or greater were pooled. The serum pool was sterilised by filtration through 0.45 μm membrane filters. The colostrum pool was treated by heating at 56° C for 2 hours and by addition of 50 μg/ml gentamicin (Roussel) and was then shown to be bacteriologically sterile.

Treatment

The eight lambs were allocated into four treatment groups of two, receiving virus and colostrum or serum as detailed in Table 1. The colostrum and serum were fed at approximately 40 ml/kg daily where indicated as part of the sterile diet of diluted condensed cows' milk divided into 3—5 meals daily according to age. The virus was given 2—4 hours after a feed.

Group 1 was intended to show if serum antibody acquired from colostrum on the first day of life would subsequently protect and group 2 to indicate whether continuous

Table 1. *Experimental design*

	Age (days)					
Group	1	2	3	4	8	9
1	C[a]	V[b]	—[c]	—	V	—
2	C	C.V.	C	C	V	—
3	—	S.[d]V.	S	S	—	V
4	—	—	—	—	V	—

[a] Colostrum
[b] Virus
[c] No treatment
[d] Serum

colostrum feeding would protect. Group 3 was included to determine if serum could be substituted for colostrum and to find if lambs made agammaglobulinaemic by feeding only milk during the first day could nevertheless be protected by the presence of antibody in the gut lumen. The challenge at days 8 or 9 was to ascertain if the previous treatments had conferred any immunity and lambs in group 4 served as controls for this challenge.

Observations

Lambs were examined clinically at least once daily, particular attention being paid to the consistency of the faeces. The voluntary milk intake was recorded. Faecal and blood samples were taken daily. The faecal samples were examined for virus and the serum samples were assayed for total IgG and screened for IF antibody against rotavirus at $^1/_{40}$ dilution (8).

Swabs were taken for bacteriological examination at intervals, and *Streptococcus faecium* and a *Micrococcus* and *Bacillus* species were isolated from different lambs. No pathogenic significance is attached to any of these bacteria.

Results

Clinical Signs

Within 24 hours of infection lambs in group 1 developed a severe diarrhoea and anorexia which was similar to that described previously for lamb rotavirus infection (8). However lambs in groups 2 and 3 remained completely normal after infection. The uninfected group 4 lambs showed no clinical signs.

When all 8 lambs were challenged at days 8 or 9 those in groups 1, 2 and 3 remained clinically normal whereas on the day following infection both control lambs in group 4 developed a transient watery diarrhoea.

Virus Excretion

No virus was detected in the faeces of any lamb before infection.

After exposure to the rotavirus on day 2 virus excretion was detected in the faeces of lambs in group 1 for up to 5 days (Table 2). In the group 2 lambs no virus excretion was detected whereas those in group 3 excreted virus on one day only.

On challenge of all 8 lambs on days 8 or 9 those lambs in group 1, 2 and 3, appeared resistant to infection, virus being detected in the faeces of one lamb on

Table 2. *Virus excretion in lamb faeces detected by IF*

		Age (days)									
Group	Lamb	2	3	4	5	6	7	8	9	10	11
1	1	−[a]	+[b]	+	+	−	+	−	−	−	−
	2	−	+	+	+	+	+	−	−	−	−
2	3	−	−	−	−	−	−	−	+	−	−
	4	−	−	−	−	−	−	−	−	−	−
3	5	−	−	−	+	−	−	−	−	−	−
	6	−	−	+	−	−	−	−	−	−	−
4	7	NT[c]	NT	NT	NT	NT	NT	−	−	−	+
	8	NT	NT	NT	NT	NT	NT	−	−	−	−

[a] Virus not isolated
[b] Virus isolated
[c] Not tested

enteritis of pigs caused by a coronavirus (Haelterman 1965).

McNulty and others (1976a) suggested that in rotavirus infections the protective role of colostrum might be quantitative, and that ingestion of sufficient colostrum to produce circulating immunoglobulin levels of over 30 mg per ml might be protective. In this paper we describe experiments to investigate the respective roles of colostrum and serum antibody in protecting lambs against diarrhoea caused by rotavirus.

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one day only. However in only one of the control lambs in group 4 was excreted virus detected after challenge.

Serum Immunoglobulin Concentrations

All 4 lambs in groups 1 and 2 had serum IgG levels on day 2 in the range 10.5 to 13.0 mg/ml. and those decreased to 5.5—9.0 mg/ml on day 11. There were no statistically significant differences between the serum immunoglobulin concentrations of lambs in groups 1 and 2 at any time during the experiment. Lambs in group 3 had less than 0.1 mg/ml serum IgG on day 2 rising to a maximum of 0.6 mg/ml on day 3 after feeding serum. This may indicate that some very slight absorption of IgG took place on day 2. IgG estimations on sera from group 4 lambs were carried out on day 8 when only low levels (<0.15 mg/ml) were present.

Serum Antibody

All 4 lambs in groups 1 and 2 had acquired serum antibody to rotavirus by absorption from colostrum by the time they were infected on day 2 and antibody was present in these lambs throughout the experiment. Lambs in group 3 had no detectable antibody until 8 days of age which was 6 days after infection. Group 4 lambs had no detectable antibody at the time of infection but antibody was present in serum 5 days later.

Discussion

The lambs in group 1 which were fed colostrum containing antibodies to rotavirus only on the first day of life were clearly susceptible to infection with rotavirus on day 2 developing clinical signs associated with virus excretion. This finding confirms the observation that calves treated in a similar manner are not protected against subsequent rotavirus infection (12). However group 2 lambs fed colostrum on the first 4 days of life and infected with rotavirus on day 2 were completely protected by the same criteria. Both groups had comparable serum immunoglobulin concentrations, and serum samples from lambs in both groups contained antibody to rotavirus at the time of infection.

Lambs in group 3 were not fed serum until day 2 at which time absorption of intact immunoglobulin from the gut is minimal (4). Consequently these lambs were virtually agammaglobulinaemic and yet were protected against infection with rotavirus. Thus circulating serum antibody appears not to be of importance in determining the outcome of rotavirus infection and protection occurs if colostrum or serum is present in the gut lumen. This may be a nonspecific effect due to a factor present in both serum and colostrum but it is more probable that it is due to the specific IgG antibody to lamb rotavirus shown to be present in both fluids. Protection attributed to the local effect of colostral and milk antibody in the gut lumen has been reported also in piglets infected with transmissible gastroenteritis virus (3).

A positive correlation between maternally-derived serum immunoglobulin in calves and resistance to diarrhoea has been suggested (5). However the diarrhoea was not rotavirus-associated in all cases and no antibody estimations were made so the results are difficult to evaluate. It is possible that with high levels of circulating immunoglobulin transfer of serum IgG to intestinal secretions from plasma may occur, as has been reported in sheep (1).

It is possible that the initial infection in groups 2 and 3 in the presence of antibody conferred immunity to subsequent challenge on days 8 or 9. Although these lambs appeared resistant on the basis of absence of clinical signs and virus excretion, the control lambs in group 4 also showed reduced response to challenge, possibly as a result of age resistance (8). However, group 3 lambs had developed detectable serum antibody by the time of challenge on day 8 so it is probable that protective local antibody was also present in the gut by this stage.

The conclusions from these experiments are that feeding of colostrum or serum containing antibody to rotavirus can protect lambs against rotavirus infection and exposure during this period of protection may immunise the lambs against subsequent infection. As most rotavirus infections in lambs and calves occur in animals less than 1 week old (10, 11), feeding of colostrum or serum would provide a rational method of protection during this period.

Acknowledgments

We would like to thank Mr. C. R. McVittie and his staff, and Miss Elizabeth Halliday and Mr. J. Menzies for technical assistance.

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Received June 1, 1976

Printed in Austria

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INFECTION AND IMMUNITY, Apr. 1977, p. 268-270

Vol. 16, No. 1
Printed in U.S.A.

Human Rotavirus in Lambs: Infection and Passive Protection

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Received for publication 19 October 1976

A human stool filtrate containing rotavirus which was administered orally to gnotobiotic lambs caused diarrhea, virus excretion, development of antibodies to rotavirus, and pathological changes in the villi of the small intestine. Thus, lambs may serve as experimental animals for the study of human rotavirus infections. This model system was used to study passive protection. Human immunoglobulin G (IgG) containing antibody to rotavirus was fed to lambs 24 to 78 h after birth, and the lambs were infected with lamb-passaged human rotavirus when 30 h old. The lambs treated with IgG did not develop diarrhea, and virus excretion was delayed in onset and shortened in duration. It may be possible to make similar use of IgG to protect children at risk in a rotavirus outbreak. The treatment did not prevent the lambs developing antibody to rotavirus.

Rotaviruses have been identified as a cause of neonatal diarrhea in several species of animals, including humans (2) and sheep (8). Neonatal piglets and rhesus monkeys have been infected successfully with human rotavirus (1, 10, 11), although attempts to infect mice, calves, and a Bonnet monkey with human rotavirus failed (1, 4, 5). In this report we describe the successful infection of gnotobiotic lambs with human rotavirus.

No prophylaxis is available for rotavirus infections in humans, although a vaccine is used to prevent calf rotavirus infections (6). However, since successful control of lamb rotavirus infections by oral administration of serum or colostrum containing antibody to rotavirus has been described (9), we investigated the possibility of controlling infection of lambs with the human rotavirus by using human immunoglobulin G (IgG) containing antibodies to rotavirus.

MATERIALS AND METHODS

Transmission of human rotavirus to lambs. Four gnotobiotic lambs were used. A stool was obtained from a 9-month-old diarrheic baby admitted to Ruchill Hospital. This child lived in an urban environment and had no known contact with any animal species in which rotavirus has been identified. Rotaviruses were observed in large numbers in the stool specimen by direct electron microscopy (EM). One part of stool was mixed with four parts of phosphate-buffered saline, and the mixture was clarified by centrifugation at 1,400 × *g* and filtered through a membrane filter (0.45-μm pore size) to give a bacteria-free filtrate, which was given orally in 5-ml amounts to lambs 1 and 2. For subsequent passage, feces collected from lamb 1 on days 2 through 6 after infection were pooled, extracted similarly, and administered orally in 1-ml amounts to lambs 3 and 4. All four lambs were infected when 2 days old.

The lambs were observed daily, with particular attention being paid to the consistency of the feces, samples of which were taken for EM examination. A 20% extract of the stool was made in phosphate-buffered saline, and the mixture was then clarified and centrifuged at 114,000 × *g* for 1 h. The pellet was suspended in 2 drops of EM diluent and mixed with an equal volume of 3% potassium phosphotungstate (pH 7), and the mixture was applied to a carbon-Formvar-coated grid for EM examination. Serum was collected from lambs 1 and 2 on day 14 after infection and was tested for antibody to rotavirus in an immunofluorescence (IF) test with calf rotavirus grown in tissue culture as the antigen (7).

Portions of small intestine were removed from lambs 3 and 4 under deep sodium pentobarbitone anesthesia on days 2 and 4 after infection, respectively, the lambs were then killed, and the intestinal contents were collected. The tissues were fixed in 1% glutaraldehyde in phosphate buffer (pH 7.4). Pieces of mucosa selected for EM examination were postfixed in osmium tetroxide and embedded in Araldite. Suitable areas for ultrathin sectioning were selected by examination of 1-μm Giemsa-stained Araldite sections. The remaining glutaraldehyde-fixed tissues were transferred to 10% buffered formalin-saline and processed for light microscopy.

Treatment of lambs with IgG. Fractions from seven batches of human normal IgG were donated by the Protein Fractionation Centre of the Scottish National Blood Transfusion Service. All were tested for rotavirus antibody by the IF test. The batch selected for subsequent use had a titer of 1:160, although all batches had a titer of at least 1:40.

Five gnotobiotic lambs (5 through 9) were fed on diluted condensed cows' milk on day 1 of life. When 24 h old, lambs 5 through 7 were each given 750 mg

enteritis of pigs caused by a coronavirus (Haelterman 1965).

McNulty and others (1976a) suggested that in rotavirus infections the protective role of colostrum might be quantitative, and that ingestion of sufficient colostrum to produce circulating immunoglobulin levels of over 30 mg per ml might be protective. In this paper we describe experiments to investigate the respective roles of colostrum and serum antibody in protecting lambs against diarrhoea caused by rotavirus.

Batch 1, pooled sera from normal sheep on two farms on which most sheep had serum antibody to rotavirus (NT 10, IgG 50·0 mg per ml); batch 2, serum from two, three month old, gnotobiotic lambs with a controlled rumen microflora (NT < 2·5, IgG 7·3 mg per ml); batch 3, serum from two sheep immunised by repeated intramuscular inoculations of lamb rotavirus (NT 3200, IgG 84·7 mg per ml). The three batches of serum were sterilised by filtration through 0·22 μm membrane filters.

of human IgG orally prior to milk feeding. This treatment was given at each feed during days 2, 3, and 4 of life, with four feeds of IgG and milk being given to each lamb per day. Each lamb therefore received a total of 9 g of IgG over the 3 days. Lambs 8 and 9 were fed only diluted condensed cows' milk. When 30 h old, all five lambs were challenged orally with 3 ml of a filtered extract of a mixture of one part intestinal content from lamb 3 with four parts phosphate-buffered saline.

The lambs were examined daily, and samples of feces were taken by rectal swabs for examination by EM. Serum samples were collected at intervals and tested for rotavirus antibody by the IF test. To check whether absorption of IgG from the gut occurred, the sera were also assayed for human IgG by using Tri-Partigen-IgG immunodiffusion plates (Behringwerke AG) with appropriate standards. In routine examination of feces, no bacteria were isolated until the 7th day of life.

RESULTS

Transmission of human rotavirus to lambs. Two days after infection with human stool filtrate, lambs 1 and 2 developed a liquid diarrhea that persisted for 3 days. No other clinical signs were apparent. Onset of diarrhea coincided with the start of rotavirus excretion, which was continuous for 6 days and sporadic for 3 additional days in both lambs. Serum collected 14 days after infection had an antirotavirus titer of >1:40.

Lambs 3 and 4 also developed diarrhea and excreted rotavirus. No macroscopic pathological changes were seen in these lambs. Under light microscopic examination, some villi in the ileum were shortened, spatulate, and infiltrated by macrophages. Ultrastructural examination showed that many epithelial cells of the small intestinal villi had shortened, fused, or deformed microvilli and contained cytoplasmic vesicles bounded by single membranes. Rotavirus-like particles were found in these cells and in subepithelial phagocytic cells (Fig. 1).

Treatment of lambs with IgG. The feces of the three treated lambs (5 through 7) were normal in color and consistency. Virus excretion was not detected until 48 h after infection and then continued for a mean of 4.6 days (range, 4 to 6 days).

At 24 h after infection, the two untreated lambs (8 and 9) appeared dull and had diarrhea. Excretion of rotavirus was detected in the feces of both lambs at this time and continued for a total of 7 days in each lamb.

Human IgG was not detected in the serum of any lamb when sampled on days 1 to 12 after infection. At the time of infection, none of the

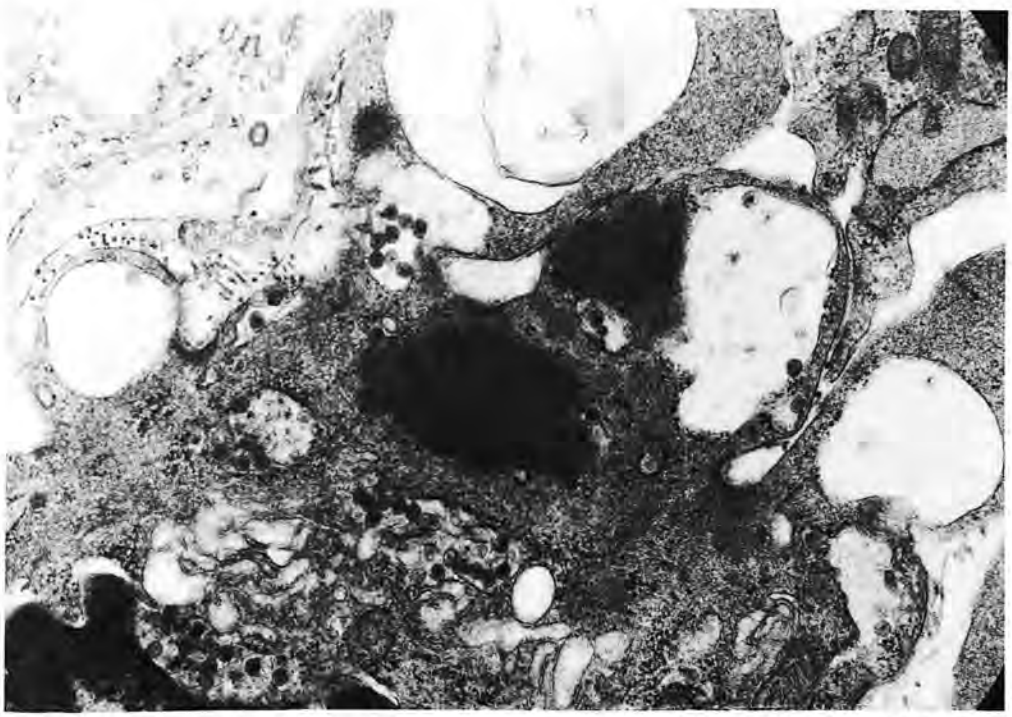

FIG. 1. *Rotavirus-like particles in phagocytic subepithelial cell in ileum from lamb 3 killed 2 days after infection with human rotavirus. Lead citrate and uranyl acetate; ×26,450.*

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five lambs had serum antibody of human or ovine origin to rotavirus by the IF test. By 12 days after infection, all lambs other than no. 5 had developed IF antibody to rotavirus.

DISCUSSION

Human rotavirus infected lambs. The production of clinical disease, the prolonged period of virus excretion, the development of antibodies, and the pathological changes detected by light microscopy and EM all suggest an active infection. Thus, gnotobiotic lambs may serve as experimental animals in the study of human rotavirus infections.

By this model system, the protective effect of human IgG given orally was assessed clinically and virologically. Clinical signs were absent from three treated lambs, whereas diarrhea occurred in all six untreated lambs. Virus excretion was delayed in onset and shortened in duration in the treated lambs, but was not reduced to the marked extent achieved in lamb rotavirus protection experiments (9). It is suggested that the presence of antibody to rotavirus in the gut partially neutralized both the initial challenge virus and virus subsequently released from infected cells. This may have reduced the level of virus infection and associated dysfunctions of absorption to the extent that no clinical disease occurred.

Human IgG is a product of plasma protein fractionation and is, at present, underutilized (J. G. Watt, personal communication). It would appear to have potential for protecting children against rotavirus infections, particularly in an outbreak in hospitalized children such as that described by Flewett et al. (3). The development of antibody in most of the protected lambs indicates that active immunity to rotavirus infections occurred by day 12. This enhances the value of the treatment, since exposure to rotavirus during the treatment may result in subsequent immunity.

ACKNOWLEDGMENTS

We thank J. G. Watt of the Scottish National Blood Transfusion Service for the supplies of human IgG, and E. W. Gray for the electron micrograph.

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Reprinted from THE VETERINARY RECORD, FEBRUARY 18, 1978

The immunoprophylaxis of rotavirus infections in lambs

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Veterinary Record (1978). **102.** 146-148

The effect of colostrum or serum feeding on subsequent rotavirus infections was investigated in neonatal gnotobiotic lambs. Immunity after feeding colostrum did not depend on absorption of passively acquired antibody into the circulation. Protection against clinical disease depended on the volume of colostrum ingested. The protection afforded by feeding serum was specific, since serum free of rotavirus antibody failed to confer protection. Immune serum fed at a rate of 2·5 ml per kg twice daily protected against rotavirus infection. Also, it was shown by intraperitoneal inoculation of immune serum that protection could occur in the absence of ingested antibody, presumably by transfer of antibody into the gut. The implications of these findings for immunoprophylaxis of rotavirus diarrhoea in lambs and calves are discussed.

THE role of maternal antibody in the protection of neonatal animals against diarrhoea caused by rotavirus has been a subject of debate due to the apparent anomaly that many infected animals have maternally derived antibody to rotaviruses in their serum (Mebus and others 1973, Woode and others 1975, Snodgrass and others 1977). Experimental evidence suggests that this serum antibody is not protective (Woode and others 1975, Snodgrass and Wells 1976). However, lambs, calves and piglets have all been protected against diarrhoea caused by rotavirus by ensuring the presence of antibody in the gut lumen (Snodgrass and Wells 1976, Bridger and Woode 1975, Lecce and others 1976). This is similar to the "lactogenic immunity" which was described as a mechanism of passive protection in transmissible gastroenteritis of pigs caused by a coronavirus (Haelterman 1965).

McNulty and others (1976a) suggested that in rotavirus infections the protective role of colostrum might be quantitative, and that ingestion of sufficient colostrum to produce circulating immunoglobulin levels of over 30 mg per ml might be protective. In this paper we describe experiments to investigate the respective roles of colostrum and serum antibody in protecting lambs against diarrhoea caused by rotavirus.

Materials and methods

Pooled intestinal contents from the fourth gnotobiotic lamb passage of lamb rotavirus (Snodgrass and others 1976) were used. A 20 per cent suspension in distilled water was filtered through 0·22 μm membrane filters and 1·5 ml of the filtrate was given orally to each of 24 gnotobiotic lambs. Virus was detected by homogenising the faeces of the experimental lambs as a 20 per cent suspension in distilled water, which was clarified by centrifugation at 1300 *g* for 10 minutes and filtered through a 0·45 μm membrane filter. The resulting filtrates were inoculated on to fetal lamb kidney (FLK) cell cultures in microtitre plates, which were then centrifuged (Snodgrass and Herring 1977). The cells were examined the following day by immunofluorescent miscroscopy for rotavirus.

Immunoglobulin G (IgG) concentrations were estimated by single radial immunodiffusion (Fahey and McKelvey 1965) with reagents prepared as described by Smith and others (1975). The neutralising titre (NT) to lamb rotavirus of serum and whey samples was determined as described by Thouless and others (1977) using FLK cells in microtitre plates (Snodgrass and Herring 1977). Titres are expressed as the reciprocal of the highest dilution giving 100 per cent neutralisation in all wells using at least three wells for each dilution.

Colostrum was collected within six hours of lambing from ewes on a farm on which most sheep had serum antibody to rotavirus. Pool 1 (NT 640, IgG 302·5 mg per ml) and Pool 2 (NT 160, IgG 93·6 mg per ml) were treated as described previously (Snodgrass and Wells 1976) and shown to be bacteriologically sterile. Serum preparations used were: Batch 1, pooled sera from normal sheep on two farms on which most sheep had serum antibody to rotavirus (NT 10, IgG 50·0 mg per ml); batch 2, serum from two, three month old, gnotobiotic lambs with a controlled rumen microflora (NT $<$ 2·5, IgG 7·3 mg per ml); batch 3, serum from two sheep immunised by repeated intramuscular inoculations of lamb rotavirus (NT 3200, IgG 84·7 mg per ml). The three batches of serum were sterilised by filtration through 0·22 μm membrane filters.

TABLE 1: Experimental design

Experiment	Lamb numbers	Day of treatment 1*	2	3	4
1	1, 2	†Pool 1, 450 ml	—	V	—
	3, 4	—	Pool 1, 450 ml	—	V
2	5, 6	Pool 2, 100 ml	—	V	—
	7, 8	Pool 2, 450 ml	—	V	—
3	9, 10	—	Batch 1, 3 × 30 ml V	Batch 1 3 × 30 ml	Batch 1, 3 × 30 ml
	11, 12	—	Batch 2, 3 × 30 ml V	Batch 2, 3 × 30 ml	Batch 2, 3 × 30 ml
	13, 14	—	Batch 3, 3 × 30 ml V	Batch 3, 3 × 30 ml	Batch 3, 3 × 30 ml
4	15, 16	—	Batch 3, 2 × 20 ml V	Batch 3, 2 × 20 ml	Batch 3, 2 × 20 ml
	17, 18	—	Batch 3, 2 × 10 ml V	Batch 3, 2 × 10 ml	Batch 3, 2 × 10 ml
5	19, 20	—	—	Batch 3, 200 ml i/p	V
Controls	21, 22, 23, 24	—	—	V	—

* day of birth = day 1
† colostrum pool or serum batch, and volume
— no treatment
i/p intra-peritoneal
V rotavirus administered

TABLE 2: Rotavirus excretion in lambs

Experiment	Lamb numbers	Time after infection (days) 1	2	3	4	5	6	7	8
1	1, 2	0	0	0	0	2*	2	1	0
	3, 4	0	0	0	1	1	0	0	0
2	5, 6	2	1	1	0	1	0	0	0
	7, 8	0	1	2	1	0	0	0	0
3	9, 10	0	2	2	2	0	1	0	0
	11, 12	2	1	1	0	1	1	1	1
	13, 14	0	0	0	0	0	0	0	0
4	15, 16	0	0	0	0	0	0	0	0
	17, 18	0	0	0	1	1	1	1	0
5	19, 20	0	0	0	1	1	0	0	0
Controls	21, 22, 23, 24	4	4	3	3	4	3]	1	0

* The results are expressed as the number of lambs excreting virus in each group on each day.

TABLE 3: Immunoglobulin concentrations and rotavirus antibody titres in lamb sera

Experiment	Lamb nos.	IgG (mg/ml)	Rotavirus antibody (NT)
1	1	28·0	40
	2	22·6	80
	3	6·0	<40
	4	3·0	<40
2	5	7·9	80
	6	7·5	40
	7	26·3	640
	8	44·6	2560
5	19	6·1	640
	20	8·3	640

EXPERIMENTAL PROCEDURES

The experimental design is shown in Table 1. Experiment 1 was designed to investigate whether a large intake of colostrum could protect, and whether protection was due to antibody in the serum or in the gut. Lambs 1 and 2 were fed colostrum from one hour after birth while lambs 3 and 4 were fed colostrum on the second day of life, at which age there is no longer significant absorption of maternal antibody (Halliday 1975, Snodgrass and Wells 1976). In experiment 2 the quantitative effect of colostrum feeding was investigated by feeding different amounts of colostrum from the same pool.

In experiments 3 and 4, serum feeding commenced on the morning of the second day of life, and continued on the third and fourth days as part of the normal diet. Lambs were infected with lamb rotavirus on day 2 at a time midway between feeds. Experiment 3 investigated the specificity of the protection afforded by using the three serum batches. In experiment 4, smaller volumes of batch 3 serum were given to find a satisfactory lower dose rate.

In experiment 5, 200 ml amounts of batch 3 serum were inoculated intraperitoneally into lambs 19 and 20 on day 3, to determine whether specific antibody in the serum was protective in the absence of ingested antibody. The lambs were infected with rotavirus on day 4. In addition, four untreated control lambs (21 to 24) were infected with rotavirus on day 3.

Lambs were examined clinically at least once daily; particular attention was paid to the consistency of the faeces and voluntary milk intake was recorded. Faecal samples were taken daily and examined for the presence of rotavirus. Blood samples were taken at intervals, and serum assayed for IgG and rotavirus neutralising antibody.

Results

All four control lambs developed severe, watery, diarrhoea on the day following infection, and two had a reduced appetite. Diarrhoea lasted for one to four days, and rotavirus was detected in faeces for five to seven days (Table 2).

The faeces of some of the lambs which were fed colostrum became looser in consistency before infection than those of the control lambs or lambs fed serum; this occurs also in colostrum fed calves (Selman and others 1970). Concentrations of immunoglobulin and titres of rotavirus antibody in the lambs' sera on the day of infection or the preceding day are given in Table 3; these waned throughout the course of the experiment.

Lambs 1 and 2 had normal concentrations of IgG and moderate titres of antibody in their sera while lambs 3 and 4 had no detectable serum antibody and very low immunoglobulin levels. However, no diarrhoea developed after rotavirus infection in any of the four lambs, and virus excretion was delayed in onset until the fourth or fifth day after infection. In experiment 2, lambs 5 and 6, which were fed the lower volume of colostrum, developed diarrhoea on the day after infection. Lambs 7 and 8, which received the larger volume of colostrum, remained normal. Rotavirus was excreted by both groups of lambs, although for short periods only.

On the day after feeding serum, all 10 lambs in experiments 3 and 4 were agammaglobulinaemic (IgG $< 0{\cdot}3$ mg per ml) and had no specific circulating antibody (NT < 40). A gradation in the protective effect of the three sera was observed; both lambs 11 and 12, which received batch 2 gnotobiotic lamb serum, developed diarrhoea. Of the lambs receiving batch 1 normal sheep serum, lamb 10 developed diarrhoea, and lamb 9 remained clinically normal. Lambs 13 and 14, which received batch 3 immune serum, continued to pass firm brown faeces throughout. Virus excretion in lambs which received batch 1 and 2 sera was not reduced significantly, but no virus excretion was detected in lambs given batch 3 immune serum (Table 2). The four lambs (15 to 18) given the reduced dosages of batch 3 serum in experiment 4 continued to pass firm brown faeces after rotavirus infection. No virus excretion was detected in lambs 15, 16 or 17, but lamb 18 excreted rotavirus for four days.

The intraperitoneal inoculation of batch 3 immune serum into lambs 19 and 20 produced low concentrations of serum IgG but high titres of specific rotavirus antibody (Table 3). Neither lamb developed diarrhoea after infection, and rotavirus was excreted for two days by lamb 19 only.

Discussion

These experiments further defined the respective roles of colostrum and serum in immunoprophylaxis of neonatal rotavirus infections. The rotavirus infected control lambs developed a transient, fluid, diarrhoea with few systemic signs of illness, similar to the experimental disease previously described (Snodgrass and others 1976). This syndrome has been consistently produced in 21 of 22 gnotobiotic lambs infected with this strain of lamb rotavirus (Snodgrass, unpublished) so any deviation from it in terms of presence or duration of diarrhoea, or in pattern of virus excretion, is significant.

In experiment 1, lambs 1 and 2, which were fed colostrum on the day of birth, had serum IgG levels within the normal range (Smith and others 1976). Lambs 3 and 4, fed equal volumes of the same colostrum on the second day, were hypogammaglobulinaemic. Specific rotavirus antibody was present in the sera of lambs 1 and 2 and absent in lambs 3 and 4. However, all four lambs were protected against diarrhoea when rotavirus was given two days after colostrum feeding. This confirms previous observations that circulating serum antibody is not essential for protection against rotavirus infections. However, it contrasts with our previous observation that feeding colostrum did not protect lambs against rotavirus infection (Snodgrass and Wells 1976). It seems probable that the protective effect of colostrum depends both on the antibody content and on the volume ingested. The latter has been suggested for calves by McNulty and others (1976a).

Experiment 2 was designed to investigate this possibly quantitative effect, eliminating the qualitative variation by using a single colostrum pool for both groups. When challenged 48 hours after infection, the lambs which had received 100 ml colostrum developed diarrhoea, while those which had received 450 ml colostrum were protected against diarrhoea, although slight virus excretion occurred in both groups. These findings confirm that the protective effect of a single dose of colostrum depends not only on the quality of the colostrum, but also on ingestion of an adequate amount.

Since there is considerable variability in the quality of colostrum and in the individual neonate and its environment, the comparison of precise volumes of ingested colostrum is difficult. However, as 100 ml colostrum was found to be insufficient to protect a 4 kg lamb, it could be suggested that 1000 ml colostrum might be insufficient to protect a 40 kg calf. This agrees with the observations of McNulty and others (1976b), which suggest from indirect evidence that a colostral dose of less than 3000 ml does not protect calves. Since few calves are likely to ingest such a volume of colostrum (Hector and Rowat 1948, Logan 1977), this may explain why the majority of colostrum fed animals remain susceptible to diarrhoea caused by rotavirus. To ensure adequate protection it seems necessary to give a very large dose of colostrum, or to continue feeding first day colostrum for several days (Snodgrass and Wells 1976).

The second part of this work was concerned with evaluating the protection afforded by feeding serum. In experiment 4 a range of effects, from susceptibility to protection, was demonstrated. Batch 2 serum (NT $< 2{\cdot}5$) did not protect lambs 11 and 12 against diarrhoea, batch 1 serum (NT 10) protected lamb 9 but not lamb 10, while batch 3 serum (NT 3200) protected both lambs 13 and 14 and completely prevented virus excretion. These findings indicate that the protective properties of serum are probably specific, and are not due to nonspecific inhibitors present in normal serum.

The potential usefulness of the immune serum was shown by the demonstration in experiment 4 of the relatively small volumes necessary to protect lambs. It is probable that 10 ml twice daily was approaching the lowest volume necessary to provide protection, as one of the lambs excreted rotavirus for several days although it remained clinically normal. Thus, immune serum feeding may be used as an alternative or a supplement to colostrum.

The final aspect studied was the possibility that passively acquired serum antibody alone might also be effective if present in sufficiently high titre. The intraperitoneal inoculation of lambs 19 and 20 with the immune serum batch 3 resulted in a high specific serum antibody titre (640) in both lambs, which was found to confer clinical protection and substantial reduction of virus excretion. Thus, contrary to what has previously been suggested, passively acquired serum antibody, while certainly not essential for the protection of neonatal lambs against rotavirus diarrhoea, nevertheless can be protective if present in high titre. This protection may result from the reverse transfer of IgG_1 antibody across the gut wall of sheep (Cripps and others 1974). This transfer may also occur in calves (Newby and Bourne 1976).

In conclusion, we suggest that colostrum can confer protection both through unabsorbed antibody remaining in the gut and through the transfer of absorbed antibody back into the small intestine.

Acknowledgements.—We are grateful to Mr G. A. M. Sharman of the Rowett Research Institute for the supply of gnotobiotic lamb serum, to Mr B. Mitchell and his staff for the supply and care of the gnotobiotic lambs, and to Mr J. Menzies and Mr M. Quirie for technical assistance.

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Reprinted from THE VETERINARY RECORD, JULY 15, 1978

Antibody titres to lamb rotavirus in colostrum and milk of vaccinated ewes

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Veterinary Record (1978) **103.** 46-48

Ewes were vaccinated two to three weeks prior to mating with a formalin-treated preparation of lamb rotavirus. The colostrum and milk produced by vaccinated ewes after the subsequent pregnancy were shown to contain significantly higher titres of antibody to the virus than did mammary secretions from non-vaccinates. The virus neutralising antibody activity was associated with IgG in both colostrum and milk. However, IgG concentrations in the mammary secretions of vaccinates and non-vaccinates did not differ. It is suggested that vaccination of the dam may be of value in protecting the suckled neonatal lamb against rotavirus infection.

Two approaches have been made to the problem of protecting neonates against diarrhoea caused by rotaviruses. Much emphasis has been placed on the development of live tissue culture attenuated calf rotavirus vaccine (Mebus and others 1973) but the efficacy of this vaccine has been questioned (Newman and others 1973, Acres and Radostits 1976). An alternative approach has been the investigation of the role of passively acquired antibody. No correlation has been observed between protection against infection and the titres of passively acquired antibody in the serum of neonates (Mebus and others 1973, Woode and others 1975, Snodgrass and others 1977). However it has been suggested that colostrum may be protective if ingested in sufficiently large quantities (McNulty and others 1976, Snodgrass and Wells 1978). In addition, the continued presence of antibody to rotavirus in the gut of neonatal lambs, calves and piglets has been shown to be important in preventing clinical disease associated with rotavirus infections (Snodgrass and Wells 1976, Bridger and Woode 1975, Lecce and others 1976). Therefore it would appear logical to attempt to stimulate the production by the dam of antibody to rotavirus in colostrum and milk, to higher titre and for a longer period after parturition. Some apparent success has been achieved by vaccinating pregnant cows with an inactivated vaccine of calf rotavirus. A reduction in the incidence of clinical diarrhoea was observed in calves born to them subsequently (Mebus and others 1973). However no measurement of the antibody titres in mammary secretions was made. An experiment was carried out to examine the influence of vaccination on the rate of decline of antibody titres and immunoglobulin concentration in the mammary secretions of ewes following parturition.

Materials and methods

Ten Cheviot ewes were treated with intravaginal tampons containing 30 mg fluogestone acetate (Synchromate; Searle) to synchronise oestrus. Two to three weeks before mating five ewes were vaccinated by intramuscular injection of 2 ml of a formalin-treated (0·5 per cent formaldehyde) lamb rotavirus preparation emulsified in an equal volume of Bayol F containing 20 per cent Falba (IFA). The rotavirus preparation was a filtrate of intestinal contents from a gnotobiotic lamb infected with lamb rotavirus at the second gnotobiotic lamb passage level (Snodgrass and others 1976). The remaining five ewes which acted as controls, were injected intramuscularly with 2 ml of a formalin-treated filtrate of intestinal contents from an uninfected gnotobiotic lamb emulsified in IFA. Blood samples were collected from all ewes prior to vaccination and at intervals during pregnancy. Serum was separated and neutralising antibody titres to lamb rotavirus were determined (Snodgrass and Wells 1978) and expressed as the reciprocal of the highest dilution giving 100 per cent neutralisation in all wells.

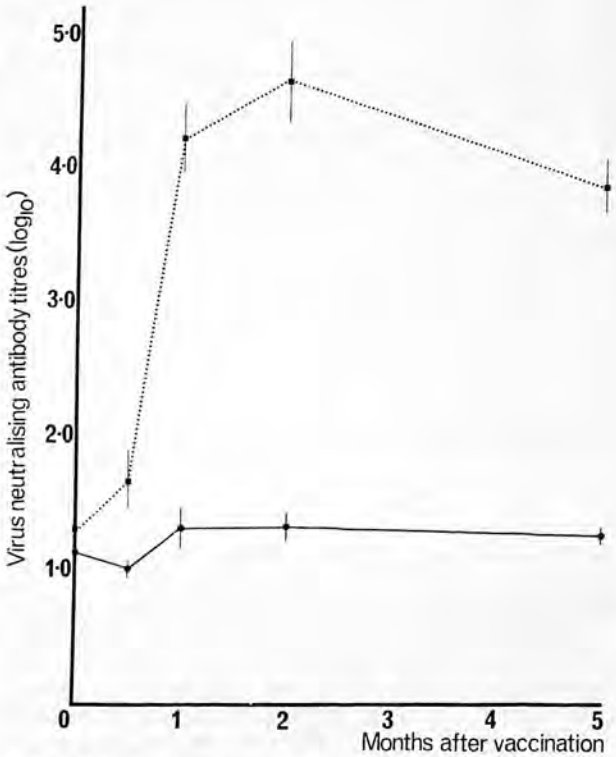

FIG 1: Geometric mean neutralising antibody titres (± SE) to lamb rotavirus in the serum of ewes following vaccination

Results

Vaccinated ewes showed a marked serological response to achieve a maximum geometric mean titre of 40,926 at two months after vaccination compared with a value of 23 in the control group at this time. Serum antibody titres in the vaccinated group were significantly different ($P<0·01$) from those in the control group from one month post-vaccination throughout gestation (Fig 1).

Colostrum was collected from each ewe within six hours of lambing and thereafter mammary secretions were sampled daily for 10 days. Whey was prepared from colostrum and milk samples by centrifugation to remove the lipids and by treatment with rennin (Smith and others 1975). Neutralising antibody titres to lamb rotavirus were determined and concentrations of IgG, IgM and IgA were measured by single radial immunodiffusion using monospecific antisera and standard immunoglobulin preparations prepared as described previously (Smith and others 1975). Geometric mean neutralising antibody titres to lamb rotavirus in the mammary secretions are shown in Fig 2. Titres of antibody to lamb rotavirus in the mammary secretions of ewes in both the vaccinated and control groups declined over the period of sampling. However the mammary secretion titres of antibody to lamb rotavirus in the vaccinated group were significantly ($P<0·01$) higher than in the control group throughout the 10 day sampling period. In contrast, although the whey samples from the vaccinated ewes contained slightly higher immunoglobulin concentrations than those collected from the ewes in the control group, no statistically significant

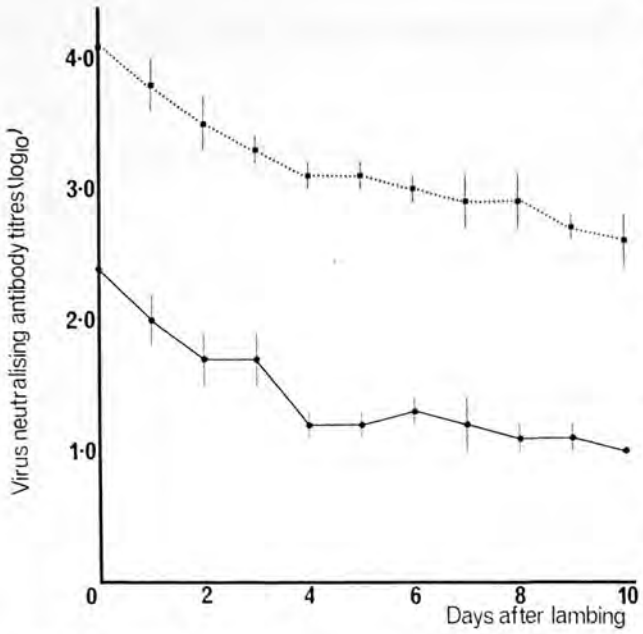

FIG 2: Geometric mean neutralising antibody titres (± SE) to lamb rotavirus in the whey of ewes following parturition

difference was observed. This result suggests that vaccination increased the concentration of specific antibody to lamb rotavirus in mammary secretion but did not greatly influence the overall concentration of immunoglobulin in the secretion.

Pooled samples of whey collected from ewes in the vaccinated group on the first, fifth and tenth days after lambing were fractionated by gel filtration through a column containing Sephadex G-200. Pooled fractions were concentrated by pressure dialysis and titrated for lamb rotavirus neutralising antibody activity. Peak antibody activity in each of the pooled samples of whey was shown to be associated with fractions containing predominantly IgG. This is the major immunoglobulin component of the colostrum and milk of sheep (Smith and others 1975) and is considered to be derived largely from the circulating serum IgG_1 (McKenzie and Lascelles 1968, Lascelles 1969). Consequently it is likely that antibody in mammary secretions is also derived from serum.

Discussion

The geometric mean titres of antibody to lamb rotavirus in the mammary secretions of the ewes given control vaccine were almost identical to those observed previously in colostrum and milk of cattle (Woode and others 1975). The present experiment has demonstrated that vaccination can result in the elevation of titres of antibody to lamb rotavirus in colostrum and milk of ewes thus prolonging the period when ingested antibody is present in the intestinal lumen. It may be possible to stimulate a similar response in cattle which may account for the success observed when pregnant cows were vaccinated with inactivated calf rotavirus (Mebus and others 1973). In support of this contention, Snodgrass and Wells (1978) report that a total daily intake of 20 ml of serum with a neutralising antibody titre to lamb rotavirus of 3200 will protect lambs against experimental infection with rotavirus. Ingestion of a comparable total amount of antibody in their daily milk intake would appear feasible in lambs being suckled by ewes producing milk with whey neutralising antibody titre of 380. This was the geometric mean antibody titre observed in whey from the vaccinated ewes 10 days after lambing.

Vaccination of the dam may be of value in protecting the suckled offspring against rotavirus infection during at least the first 10 days of life. Experimental evidence indicates that in the likely event of natural exposure to the virus during this period active immunity may develop to protect against subsequent infection (Snodgrass and Wells 1976). It is probable that an inactivated vaccine of the tissue culture adapted calf rotavirus might be practicable and of value as suggested previously by Mebus and others (1973).

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Printed by H. R. GRUBB LTD., Croydon, Surrey.

Reprinted from the JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION, Vol.173, No.5, Pages:565-568

Passive Immunity in Rotaviral Infections

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ONE OF the most important aspects of rotaviral infections is the problem of their prevention. This is particularly intriguing because animals in need of protection are those in the first few days and weeks of life. Active immunization of the newborn animal is one possible approach to this problem, and a tissue culture attenuated calf rotaviral vaccine is available.[1] However, the efficacy of this vaccine in the field has been questioned.[2,3] We decided to investigate the alternative approach of passive immunization.

Mechanisms by which immunity to a neonatal viral enteritis can be transmitted from dam to offspring have been studied in transmissible gastroenteritis (TGE) of pigs.[4-7] In TGE, the term "lactogenic immunity" was used to describe the protection conferred by the continuing presence of antibody in the gut. It was assumed that immunity depended on neutralization of virus in the lumen of the alimentary tract by antibody in colostrum or milk.[6] The most effective protection was provided by the continued secretion of immunoglobulin A (IgA) in milk, although immunoglobulin G (IgG) has been shown to be protective when in high concentrations.[4,5,7]

Passive immunity may be provided by stimulating the dam to secrete antibody in colostrum and milk. Alternatively, for animals not suckling, passive immunity may be provided by artificial feeding of the neonate with antibody-containing preparations. We have pursued both these approaches, the information gained from the initial feeding studies being of value to subsequent vaccinal studies.

Our work has been undertaken primarily in gnotobiotic lambs infected with lamb rotavirus. Lambs 2 to 5 days old, infected orally with a 0.22 μm filtrate of gnotobiotic lamb intestinal contents containing rotavirus, consistently developed diarrhea and small intestinal lesions.[8,9] The laboratory techniques used have been described.[10]

Role of Colostrum

Most adult domestic animals have serum antibody to rotavirus. In cattle and sheep, this antibody is secreted in high concentrations in 1st-day colostrum and decreases rapidly to negative amounts within 3 days after parturition.[11,12] Many neonatal animals infected with rotavirus have circulating rotaviral antibody absorbed from this colostrum.[1,12,13] This has naturally caused the protective role of colostrum to be questioned, although a relationship between resistance to rotaviral infection and high colostral intake in calves has been suggested.[14] Similarly in man, the presence of preexisting serum antibody to rotavirus does not correlate with resistance to infection in adults or children.[15,16]

TABLE 1—Colostrum Feeding Experiments—Pool 1

Lamb No.	Age (days) 1*	2	3	4	IgG (mg/ml)	Rotaviral antibody (NT)	Clinical result
1	100 ml	....	V	....	7.9	80	Diarrhea
2	100 ml	....	V	....	7.5	40	Diarrhea
3	450 ml	....	V	....	26.3	640	Normal
4	450 ml	....	V	....	44.6	2,560	Normal

* Day of birth = day 1.
.... = No treatment; V = rotavirus given orally; NT = neutralization titer.

TABLE 2—Colostrum Feeding Experiments—Pool 2

Lamb No.	Age (days) 1*	2	3	4	IgG (mg/ml)	Rotaviral antibody (NT)	Clinical result
5	450 ml	....	V	....	28.0	40	Normal
6	450 ml	....	V	....	22.6	80	Normal
7	....	450 ml	....	V	6.0	< 40	Normal
8	....	450 ml	....	V	3.0	< 40	Normal

* Day of birth = day 1.
.... = No treatment; V = rotavirus given orally; NT = neutralization titer.

We have attempted to elucidate this situation experimentally (Table 1).[10,17] Two lambs (No. 1 and 2) were fed 100 ml of a colostrum pool on the 1st day of life. In spite of moderate serum neutralizing antibody concentrations, they were susceptible to infection when challenged with rotavirus on the 3rd day, developing diarrhea and excreting virus. This observation paralleled the findings in natural and experimental disease in calves.[12] However, as it has been suggested that the effects of colostrum on susceptibility to rotavirus infection might be quantitative,[14] 2 other lambs (No. 3 and 4) were fed 450 ml of the same colostrum pool and were challenged with rotavirus on the 3rd day (Table 1). They remained clinically normal, thus demonstrating that colostrum can protect against rotaviral infections if fed in large enough quantities. However, such protection may well be transient, and the animal may be susceptible to rotaviral diarrhea if challenged at intervals of longer than 48 hours after colostrum feeding.

This experiment did not indicate the mechanism whereby the large volumes of colostrum conferred protection, whether it be due to the residual presence of antibody in the gut or to circulating antibody. In an attempt to answer this point, a 2nd colostrum pool was fed to 4 lambs at the higher dose of 450 ml, to 2 of them (No. 5 and 6) within a few hours of birth, and to the 2 others (No. 7 and 8) at 24 hours old (Table 2). Lambs 5 and 6 subsequently had serum IgG values within the normal range, but lambs 7 and 8 were hypogammaglobulinemic. Similarly, specific rotaviral anti-

TABLE 3—Colostrum Feeding Experiments—Pool 3

Lamb No.	Age (days) 1*	2	3	4	IgG (mg/ml)	Clinical result
9	100 ml	100 ml, V	100 ml	100 ml	12.5	Normal
10	100 ml	100 ml, V	100 ml	100 ml	11.0	Normal
11	100 ml	V			13.0	Diarrhea
12	100 ml	V			10.5	Diarrhea

* Day of birth = day 1.
= No treatment; V = rotavirus given orally.

TABLE 4—Viral Excretion and Disease in Lambs Fed Serum

Serum	Lamb No.	Age (days) 2	3	4	5	6	7	8	9	10	Clinical result
Hyperimmune (NT 3,200)	13	–	–	–	–	–	–	–	–	–	Normal
	14	–	–	–	–	–	–	–	–	–	Normal
Normal (NT 10)	15	–	–	+	+	+	–	–	–	–	Normal
	16	–	–	+	+	+	–	+	–	–	Diarrhea
Negative (NT < 2.5)	17	–	+	+	–	–	–	+	+	+	Diarrhea
	18	–	+	–	+	–	+	–	–	–	Diarrhea

NT = Neutralization titer; + = virus shed; – = virus not shed.

body was in the sera of lambs 5 and 6 and was absent in lambs 7 and 8. However, all 4 lambs were protected against diarrhea when challenged with rotavirus 2 days after colostrum feeding, and viral excretion was delayed for several days. These results suggest that serum antibody is not essential for protection against rotaviral infection, but that antibody needs to be in the gut lumen for effective protection. This situation is analogous to the lactogenic immunity of TGE infections.

It was possible to confirm the importance of antibody in the gut by continuing to feed lambs 9 and 10 with 1st-day colostrum at the lower dosage of 100 ml daily for 4 days and challenging during this period (Table 3). This resulted in clinical protection and complete elimination of virus excretion. As controls, lambs 11 and 12, which were given this colostrum pool on the 1st day only, were susceptible to rotaviral infection. Protection by continued feeding of colostrum also has been shown in calves.[18]

Thus, there appear to be 2 ways in which colostrum can be used to confer protection against rotaviral diarrhea: either by ensuring ingestion of large amounts of colostrum for a short period only, as happens naturally in suckled animals, and which protects for at least 48 hours; or, possibly more effectively, by continuing to feed smaller amounts of colostrum as part of the milk diet, which can presumably protect for as long as it is continued.

In addition to specific antibodies in colostrum and milk, nonspecific viral inhibitors also may be present. Antiviral activity in a nonantibody-containing glycoprotein fraction of human and bovine milk has been shown.[19] This same glycoprotein fraction from cows' milk has antirotaviral action.[20] Its in vivo importance has not been assessed.

Use of Serum and Serum Fractions

The effect of feeding serum on the course of rotaviral infections also was investigated.[10] Serum antibodies offer a controllable model for antibodies in colostrum, and it is also possible that serum or serum products may themselves be a feasible method for rotaviral phophylaxis or therapy. Our standard method of assessing sera involved serum feeding starting on the morning of the 2nd day of life. The dose (30 ml) was given before the milk feeding, and this dose was continued 3 times daily on the 2nd, 3rd, and 4th days of life. All lambs remained agammaglobulinemic under this treatment. Lambs were infected with rotavirus at a time midway between feedings on the 2nd day of life.

We have used 3 serum pools in this regimen: a hyperimmune sheep serum (neutralization titer (NT), 3,200); a normal adult sheep serum pool (NT, 10); and a serum pool obtained from gnotobiotic lambs (NT, < 2.5). Results (Table 4) demonstrate a progression from absence of disease and elimination of viral excretion in the group treated with hyperimmune sheep serum, to a normal experimental rotaviral diarrhea in the group treated with negative serum. This suggests that the protective action of immune serum is specific and is probably mediated by the major immunoglobulin class present, ie, IgG. By analogy, it is probable that normal colostrum, which contains high concentrations of rotaviral antibody[11,12] also protects largely through specific antibody.

The volume of serum used in these experiments was higher than would be feasible to use as a practicable method of prophylaxis. We therefore used reduced dosages of the hyperimmune serum, from three 30-ml feedings daily, to 2 of 20 ml, and 2 of 10 ml daily. These lower dosages conferred complete clinical protection, but at the lowest dose, slight viral excretion occurred. This may indicate that 10 ml, given twice daily, was approaching the end point of effective protection. Thus, hyperimmune serum may provide a method for protecting neonatal animals against rotaviral diarrhea.

A similar approach has been made in the prophylaxis of experimental rotaviral infection of pigs.[21] It was shown that porcine γ globulin incorporated in the milk diet substantially alleviated clinical signs, but porcine albumin had no effect. This demonstrated the specific nature of the protection afforded by serum.

A further development in the use of serum was stimulated by the transmission of human rotavirus to lambs, producing viral excretion and diarrhea.[22] We obtained several batches of normal human IgG from the Scottish National Blood Transfusion Service, and all were found to contain antibody to rotavirus. We therefore fed lambs with 1 batch of human IgG under a 3 times a day regimen similar to that already described, and infected them with human rotavirus. Our treated lambs remained clinically normal, but the untreated lambs developed diarrhea. Viral excretion also was delayed and reduced in the treated animals. Thus, specific passive protection may have a role in human rotaviral infection because the human IgG product used is a by-product of plasma protein fractionation and is produced in substantial excess.

In this series of serum feeding experiments, circulating antibody was not detected in any lamb, and the importance of antibody in the gut was established. However, it may be premature to completely ignore the possible importance of circulating antibody. We have inoculated lambs intraperitoneally with 200 ml each of

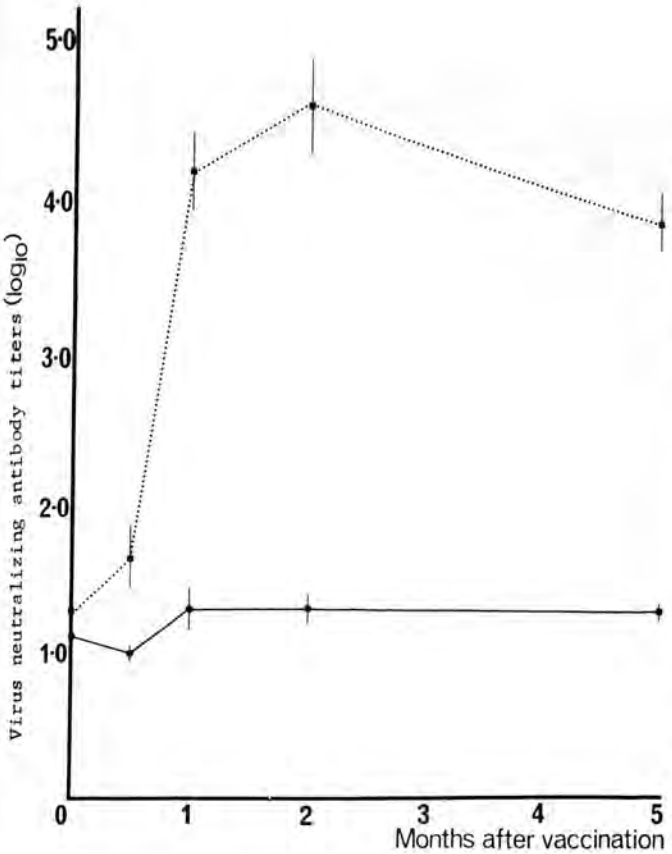

Fig 1—Geometric mean neutralizing antibody titers (± SE) to lamb rotavirus in the serum of ewes following parturition. . . . Vaccinates; — controls. (Reproduced by permission of the *Veterinary Record.*)

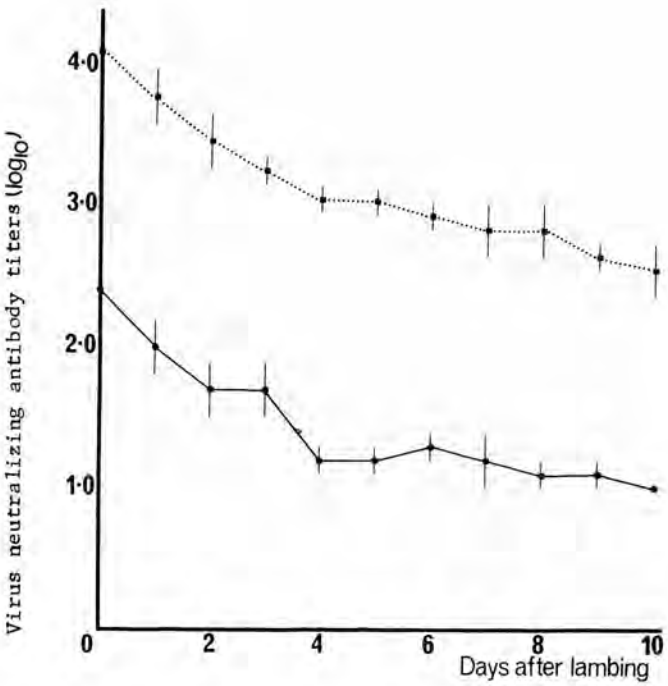

Fig 2—Geometric mean neutralizing antibody titers (± SE) to lamb rotavirus in the serum of ewes following vaccination. . . . Vaccinates; — controls. (Reproduced by permission of the *Veterinary Record.*)

hyperimmune serum, producing high circulating antibody concentrations without directly exposing the gut to antibody.[10] On infection with rotavirus, these lambs did not develop diarrhea, and rotaviral excretion was substantially reduced. This may be due to a transfer of antibody from the circulation to the gut lumen.[23] Thus, although the presence of circulating passively acquired antibody does not correlate with protection in most cases, nevertheless, it may under certain circumstances be protective.

Inoculation of the Dam

If a dam could be stimulated to produce rotaviral antibodies in its colostrum and milk for a prolonged period after parturition, this would ensure continued antibody in the gut of the suckling neonate. Thus, the protective situation that we have produced experimentally would be achieved. Unfortunately, although specific rotaviral antibody is in high titer in 1st-day colostrum of normal cows, it is absent within 3 days after parturition.[12] This is associated with the rapid decline in total immunoglobulins in cows' colostrum and milk after parturition.[24]

Inoculation of cows with inactivated calf rotavirus has been reported.[1] This resulted in a reduced incidence of diarrhea in their calves, but milk antibody estimations were not made. We have inoculated ewes prior to mating with inactivated lamb rotavirus emulsified in Freund's incomplete adjuvant.[11] These animals and a similar number of controls were then mated, and the serum antibody response was monitored. There was a highly significant ($P = < 0.01$) increase in antibody titer which continued throughout pregnancy (Fig 1).

Colostrum samples were collected from all ewes within 6 hours of lambing, and milk samples daily thereafter for 10 days. Mean neutralizing antibody titers to lamb rotavirus in the whey of the mammary secretions are shown in Figure 2. The control ewes had moderate titers of antibody in 1st-day colostrum that were undetectable by the 4th day. The vaccinated ewes had high antibody titers in 1st-day colostrum, and these remained significantly higher ($P < 0.01$) than the titers in the control group throughout the 10 day sampling period. There was no significant difference in the whey immunoglobulin concentrations of the vaccinated and control groups, so the raised titer was due to an increase in specific antibody. In both colostrum and milk, the antibody activity was shown to be associated largely with IgG.

This experiment demonstrated that vaccination could result in the elevation of titers of antibody to lamb rotavirus in colostrum and milk of sheep. We have shown that a total daily intake of 20 ml of serum with a neutralizing antibody titer of 3,200 will protect lambs against experimental rotaviral infection.[10] Clearly, a much greater total daily intake of antibody would occur in lambs suckling ewes producing milk with a neutralizing antibody titer of 380, which was the mean titer observed in the vaccinated group of ewes 10 days after lambing. Thus, this vaccination is likely to prolong the period over which the neonate is protected by the pres-

ence of ingested antibody in the gut lumen. It may be possible to stimulate a similar response in cattle, and this may account for the success obtained when pregnant cows were inoculated with an inactivated calf rotaviral vaccine.[1]

Conclusions

We have used the lamb infected with lamb rotavirus as a model for the study of passive immunity in rotaviral infections. Clinical protection can be achieved while allowing a reduced degree of viral multiplication, which may be desirable because it could result in a subsequent state of active immunity.[17,22] Passive immunity may have a useful role in the prophylaxis of lamb rotaviral infections, and it is possible that the conclusions may apply equally to other species.

Our results emphasize the importance of the traditional husbandry practice of feeding colostrum in as large amounts as possible, but in addition, suggest that more effective protection may be achieved by continuing to feed 1st-day colostrum as part of the diet throughout the period of greatest risk. It also may be possible to develop serum or serum products as substitutes for colostrum. Vaccination of the dam may be especially useful in protecting neonates against rotaviral infection through improving the antibody content of colostrum and milk.

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INFECTION AND IMMUNITY, May 1980, p. 344-349
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Vol. 28, No. 2

Passive Immunity in Calf Rotavirus Infections: Maternal Vaccination Increases and Prolongs Immunoglobulin G1 Antibody Secretion in Milk

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Ten heifers were inoculated on two occasions with an inactivated preparation of tissue culture-grown calf rotavirus, and a further ten heifers received a placebo vaccine. Serum anti-rotavirus antibody titers were significantly increased throughout pregnancy in the vaccinated group. After calving, the mean neutralizing antibody titer of colostral whey in control cows was 100, associated with immunoglobulins A and G1. No antibody was detected in the milk of these cows after the 4th day postpartum. The colostral whey from the vaccinated cows had a mean antibody titer of 20,452; 28 days after calving, the mean milk antibody titer was 320, associated mainly with immunoglobulin G1. Calves were challenged with a large oral inoculum of calf rotavirus at the 7th day of age. There was significant lengthening of the incubation and prepatent periods in calves born to vaccinated dams, but rotavirus-associated diarrhea of equal severity occurred in both groups. Evidence is presented which suggests that rotavirus antibody in milk can protect against a smaller challenge dose. Maternal immunization against rotavirus may be a practical proposition.

It has been shown experimentally that passively acquired antibodies from either serum or colostrum can protect young animals against diarrhea caused by rotavirus infections (2, 4, 12).

The practical exploitation of this passive protection is to stimulate the dam to produce in her colostrum and milk high titers of antibodies to rotavirus. Maternal vaccination has been used to protect piglets against transmissible gastroenteritis (1, 9), but has not been used in enteric virus infections of ruminants. In a preliminary experiment in sheep, titers of milk antibodies to rotavirus were successfully elevated for the first 10 days after lambing (14). The work reported in this paper aimed to repeat that experiment in cattle, to follow milk antibody for a longer period and in more detail, and to observe the effects of rotavirus infection on calves born to vaccinated and control dams.

MATERIALS AND METHODS

Animals. Ten Hereford × Friesian and ten Blue-Grey (Shorthorn × Galloway) 2-year-old heifers were allocated to two comparable groups by breed and preexisting serum neutralizing antibody titers to rotavirus. Ten heifers were vaccinated and ten received a placebo vaccine. All were mated naturally commencing 2 weeks after initial vaccination. They were revaccinated in a similar manner 7 months later, approximately 2 to 3 months before calving. Nine control and seven vaccinated heifers produced live calves at term.

Immediately after calving, cows and their calves were moved to separate clean accommodation. On the 7th day postpartum, each cow and her calf were again moved to separate housing, and the calves were infected orally with calf rotavirus. No contact was allowed between this postchallenge group and the animals at earlier stages of the experiment. A harness for total fecal collection was put on all male calves on the 7th day (seven bull calves born to control cows and three born to vaccinated cows).

Rotavirus. For vaccine preparation, tissue culture-adapted calf rotavirus was used (2). Virus was treated with trypsin (10 μg/ml) for 1 h at 37°C and then was inoculated onto bovine embryo kidney (BEK) cells with trypsin (10 μg/ml) included in the maintenance medium (13). Rotavirus at the sixth and ninth passage in our laboratory was used for the first and second vaccination, with titers prior to inactivation of $10^{4.8}$ and $10^{7.8}$ $TCID_{50}$ (50% tissue culture infective doses) per ml, respectively. The virus was inactivated by overnight incubation with 0.5% formaldehyde at 4°C. Equal volumes of calf rotavirus and incomplete Freund adjuvant (Difco Laboratories) were emulsified, and 2.0 ml of the emulsion was inoculated by deep intramuscular injection into the neck. The placebo vaccine was identically treated control BEK cultures.

The challenge calf rotavirus was intestinal contents from the eighth gnotobiotic calf passage, obtained from J. C. Bridger, Institute for Research on Animal Diseases, Compton, England. Volumes of 2 ml of in-

testinal contents containing 10^{11} particles per g, diluted in 10 ml of phosphate-buffered saline, were used as an oral challenge in calves. The vaccine and the challenge rotavirus were both derived from the same origin (2). No virus other than rotavirus was detected by electron microscopic examination of this inoculum.

Observations. Each heifer was bled for serum at intervals throughout pregnancy, at parturition, and 28 days later. Colostrum and milk samples were collected on 1, 2, 3, 4, 6, 8, 10, 14, 21, and 28 days after calving, the first sample being obtained within 8 h of calving, and where possible before the calf had sucked. Samples of feces were collected. Total feces collected from the postchallenge bull calves were examined daily for total fecal output and were dried to constant weight for dry-matter estimation. All calves were examined clinically at least once a day.

Neutralization test. Serum samples, and whey from colostrum and milk samples, were tested for the presence of neutralizing antibody to tissue culture-adapted calf rotavirus on BEK cells or Vero cells grown in microtiter plates (11). Titers are expressed as the reciprocal of the highest dilution giving complete neutralization.

Feces examination. Fecal samples were examined for rotavirus by counterimmunoelectroosmophoresis (6). The antiserum used was prepared by inoculation of rabbits with calf rotavirus purified by centrifugation on cesium chloride density gradients.

Additional fecal samples were taken at least once from each calf while scouring and were examined by electron microscopy to detect rotavirus and other viruses. At the same time, the feces were examined bacteriologically. Three *Escherichia coli* isolates from each calf were grown in Minca medium (3) and tested for the presence of K99 antigen by slide agglutination.

Fractionation and analysis of whey. Equal volumes of whey from individual vaccinated cows on the 1st, 3rd, 6th, 14th, and 28th days after calving were pooled. Whey from control cows was similarly pooled on the 1st and 3rd days after calving. Two milliliters of pooled whey from day 1 or 5 ml of whey obtained at the other times was loaded onto a 2.6 by 82 cm column of S300 (Pharmacia Fine Chemicals, Inc.) and eluted with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride–1.0 M NaCl buffer (pH 8.0) at 16 ml/h. Every other 6-ml fraction was concentrated to 2 ml by dialysis against Carbowax PEG, 20 M (Union Carbide Corp.), and phosphate-buffered saline. The fractions were assayed for both virus neutralizing antibody and the class and relative concentration of immunoglobulin. Immunoglobulins were analyzed by single-radial immunodiffusion using monospecific rabbit anti-sheep immunoglobulin sera (10), which cross-reacted with the respective bovine immunoglobulins. In the absence of standard bovine immunoglobulin preparations, the results are calculated and displayed as the relative concentrations of each immunoglobulin in the fractions.

Fractions from the immunoglobulin G (IgG) region of the S300 whey fractionation from the control cows on day 3 and from the vaccinated cows on days 3 and 14 were pooled, concentrated, dialyzed, and loaded onto a 1.5 by 25 cm column of DE52 (Whatman, Inc.) and eluted with 0.01 M phosphate buffer, pH 7.6, and then 0.03 M NaCl–0.01 M phosphate buffer, pH 7.6, at 30 ml/h. The eluates from each step were pooled, concentrated, and assayed for antibody activity. The pools were analyzed for immunoglobulin class by immunoelectrophoresis using monospecific anti-sheep immunoglobulin sera.

RESULTS

Effect of vaccination on serum antibody. Vaccination significantly raised the serum neutralizing antibody titers of the heifers from a geometric mean titer of 63 on the day of vaccination to a peak titer of 11,910 by 1 month after initial vaccination (Fig. 1). These titers waned throughout pregnancy and were increased by revaccination to a mean titer of 3,929. Immediately prior to parturition, at 9 months after vaccination, the mean titers in the vaccinated and control group were 3,929 and 74, respectively.

Effect of vaccination on milk antibody. Whey prepared from the first colostrum sample from control cows had a geometric mean neutralizing antibody titer of 100 (Fig. 2). No anti-rotavirus antibody was detected in whey from control cows after the 4th day of milking. First colostrum whey from vaccinated cows had a mean antibody titer of 20,452, which by 28 days after calving had declined to 320.

Analysis of colostral and milk antibody. Fractionation of whey obtained from control cows on the day of calving showed the peak antibody activity in fractions containing mainly IgA and IgG1, the titer decreasing in fractions containing IgG1 alone (Fig. 3). In vaccinated cows the titer of antibody in fractions of day 1

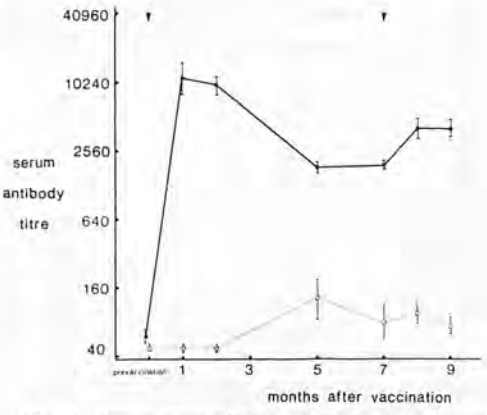

FIG. 1. *Serum neutralizing antibody titers (mean ± standard error) of rotavirus-vaccinated heifers (solid line) and placebo-vaccinated heifers (dotted line). Arrows indicate times of vaccination.*

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Conclusions

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whey containing IgA and IgG1 was substantially increased, although the peak antibody activity appeared in later fractions, some of which contained only IgG1 (Fig. 4). IgG2 was not detected in any of the fractions.

By 14 to 28 days after calving, the antibody activity in the milk from vaccinated cows was mainly in the IgG1 region of the elution profile. This was very pronounced by day 28, at which time antibody activity was almost exclusively associated with IgG1 (Fig. 5).

Fractionation of the IgG region from the S300 columns by anion-exchange chromatography confirmed the very low titers of IgG2 antibody in whey obtained 3 and 14 days after calving and demonstrated that activity was in the fraction containing IgG1 (Table 1).

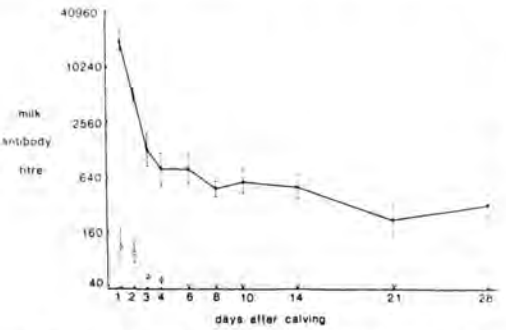

FIG. 2. *Neutralizing antibody titers (mean ± standard error) in whey from colostrum and milk of vaccinated (solid line) and control (dotted line) heifers after calving.*

Effect of vaccination on rotavirus infection in calves. After challenge, all nine calves born to control cows were observed to develop diarrhea, after a mean incubation period of 3.0 days (Table 2). Five of these calves became dull and clinically dehydrated, one requiring oral fluid replacement therapy. Most calves scoured for 3 to 5 days, and all recovered and subsequently thrived. Five of the seven calves born to vaccinated cows also developed diarrhea, after a mean incubation period of 4.8 days. Four of these five became dull, and the severity of reaction was indistinguishable from that of the controls.

Effect of vaccination on virus excretion. No virus excretion was detected by counterimmunoelectroosmophoresis in any calf before challenge. Rotavirus excretion was detected after infection in all calves born to control cows, and in six of the seven born to vaccinated cows. The prepatent period to virus excretion in the vaccinated calves was 5.2 days compared with 2.0 days for the controls ($P < 0.01$) (Table 2). There was no difference between the two groups in duration of virus excretion. No virus other than rotavirus and no pathogenic bacteria or *E. coli* with K99 antigen were detected in any calf.

Effect of vaccination on feces measurement. The weight of feces produced by the bull calves increased from a normal level of less than 200 g daily to a mean maximum after infection

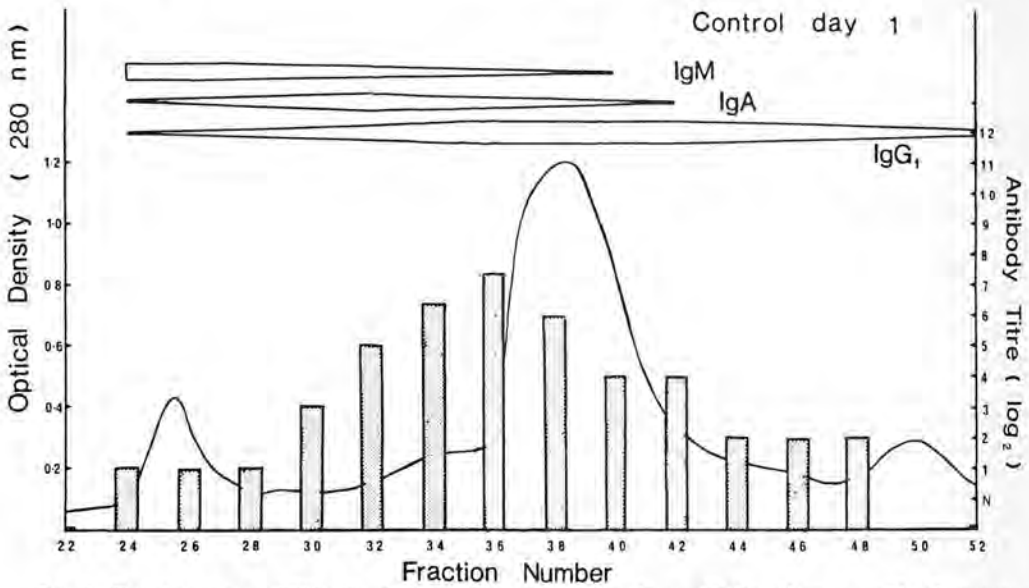

FIG. 3. *Fractionation on S300 of pooled whey from day 1 control cows. Optical density profile. Histograms: neutralizing antibody titer. Bars: relative immunoglobulin concentrations.*

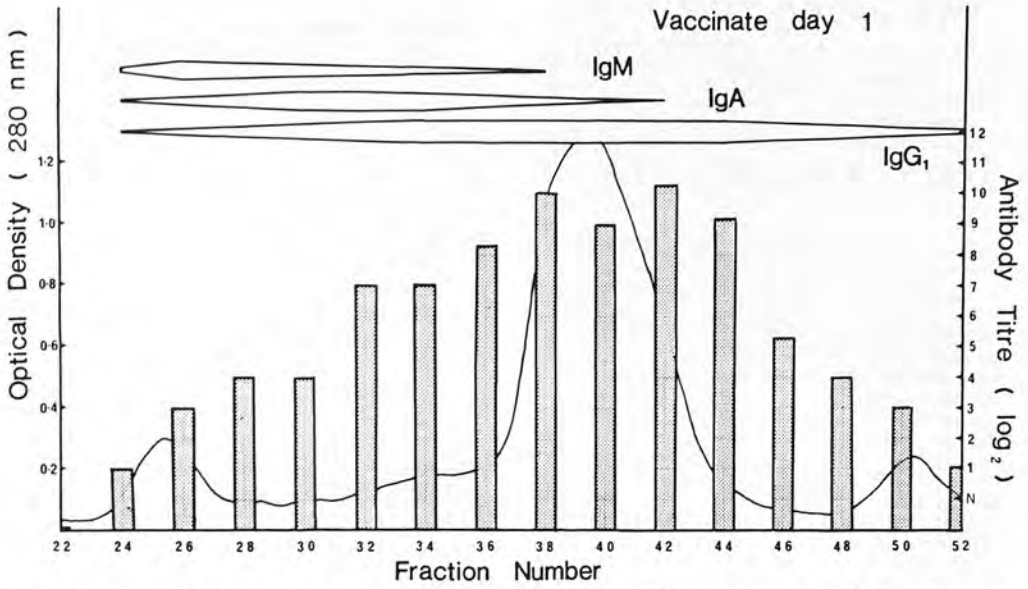

FIG. 4. *Fractionation on S300 of pooled whey from day 1 vaccinated cows. Optical density profile. Histograms: neutralizing antibody titer. Bars: relative immunoglobulin concentrations.*

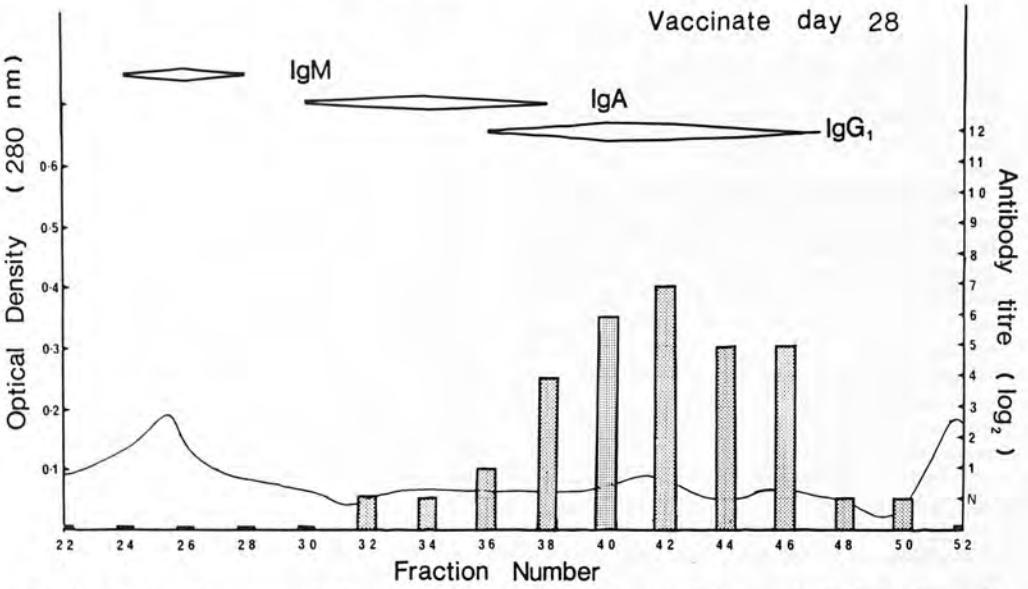

FIG. 5. *Fractionation on S300 of pooled whey from day 28 vaccinated cows. Optical density profile. Histograms: neutralizing antibody titer. Bars: relative immunoglobulin concentrations.*

of 1,888 g in the control calves and 1,868 g in the vaccinated calves (Table 2). The dry-matter content of the feces before infection was greater than 20%, and this fell to a mean minimum of 12% in both groups. There were significant delays in the increase in fecal weight and decrease in dry matter (1.8 days) in the vaccinated calves compared with the controls ($P < 0.01$).

DISCUSSION

Vaccination of the heifers with an inactivated calf rotavirus preparation significantly increased

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Conclusions

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TABLE 1. *Antibody titers in DE52 fractions of bovine IgG*

Source	Original 7S pool	IgG2 pool	IgG1 pool
Control, day 3	32	4	64
Vaccinate, day 3	2,560	64	2,048
Vaccinate, day 14	256	16	256

TABLE 2. *Reaction of calves to rotavirus challenge*[a]

Parameter	Controls	Vaccinates	Significance
No. with diarrhea	9/9	5/7	NS
No. clinically dull	5/9	4/7	NS
Days virus detected	3.7 ± 0.5	3.0 ± 0.9	NS
Maximum fecal output (g/day)	1,888 ± 146	1,868 ± 96	NS
Minimum fecal dry matter (%)	11.8 ± 1.9	11.7 ± 1.2	NS
Onset of diarrhea (days)	3.0 ± 0.5	4.8 ± 0.7	$P < 0.01$
Onset of virus shedding (days)	2.0 ± 0.3	5.2 ± 0.9	$P < 0.01$

[a] The first five parameters relate to severity of reaction; the last two, to timing of reaction. Mean ± standard error. NS, Not significant.

serum antibody titers throughout pregnancy. There was also a marked effect on anti-rotavirus milk antibody levels during subsequent lactation, with high titers being detected in clostrum and milk throughout the 28-day observation period.

Although colostrum from control heifers contained anti-rotavirus antibody, which was largely IgA, no specific antibody was detected after the first 4 days of lactation. Porter (8) made similar observations on anti-*E. coli* antibody in cows' milk. IgA antibody was also present at similar or elevated levels in colostrum from the vaccinated heifers, but in addition their colostrum contained high titers of IgG1 anti-rotavirus antibody. From 2 days after calving, the anti-rotavirus antibody detected in the milk of the vaccinated heifers was predominantly IgG1, this antibody arising by selective transfer from serum (7). IgG2 was present in milk at very low concentrations and contained little antibody activity.

As rotavirus infections are endemic in cattle, most adult cows will have been naturally infected. The sustained antibody response in serum and milk of vaccinated cows may have been due, in part, to this primary gut exposure prior to systemic vaccination (1).

The effect of increased milk antibody on rotavirus diarrhea in the calves was to delay the establishment of infection, but not to reduce its ultimate severity. The lengthened lag phase suggests that the greater part of the inoculum was neutralized before it could infect the gut, but that sufficient viable virus survived to infect the calves subsequently. The lag and wide variability of rotavirus shedding by the vaccinates, as compared to the controls, was also noted. This is possibly due to the continuous ingestion of high levels of milk antibody by the calf of a vaccinated cow which neutralizes the virus resulting from the infection. Eventually, the amount of virus produced in the gut exceeds the amount of antibody ingested, which is then observed in the feces. Alternatively, the anorexia, which often accompanies diarrhea, reduces the uptake of milk antibody by the calf. Once this happens, there would be very little difference between calves suckling vaccinated or control cows. A high challenge dose of virus was chosen deliberately, as difficulties were expected in establishing rotavirus infections in conventional sucking calves (5). It is unlikely that calves under farm conditions will ingest at any one time a 2-g bolus of feces containing a high titer of rotavirus. Calves sucking vaccinated cows may have been protected against a smaller challenge of rotavirus.

Direct evidence in support of this view comes from experiments with lamb rotavirus (K. J. Fahey and D. R. Snodgrass, unpublished data). Pooled milk taken from six control ewes 6 days after lambing was fed to two gnotobiotic lambs which, after rotavirus challenge, developed diarrhea and excreted rotavirus. By contrast, pooled milk taken from five vaccinated ewes (14) 6 days after lambing completely protected two gnotobiotic lambs against clinical and virological signs of rotavirus infection. This experiment demonstrated that the titer of antibodies present in milk obtained from vaccinated dams 6 days after parturition was sufficient to protect completely against a moderate challenge dose of rotavirus. This emphasizes the importance of using carefully titrated moderate challenge inocula in passive immunization experiments, where only a finite amount of antibody and hence protection can be present.

It is postulated that the technique of dam vaccination against rotavirus will stimulate IgG1 neutralizing antibodies in the circulation of the dam which are passively transferred to the milk for a substantial period after calving, and which may protect calves against natural infection under field conditions.

ACKNOWLEDGMENTS

We thank Janice Bridger for the gnotobiotic calf intestinal contents, A. Dawson for fractionating bovine wheys, and M. McLauchlan for the statistical analyses. Technical help was received from Margaret Gordon and help in handling the cattle, from G. Newlands.

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Veterinary Immunology and Immunopathology, 2 (1981) 27–33
Elsevier Scientific Publishing Company, Amsterdam – Printed in Belgium

IgG_1 ANTIBODY IN MILK PROTECTS LAMBS AGAINST ROTAVIRUS DIARRHOEA

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(Accepted 6 November 1980)

ABSTRACT

Fahey, K.J., Snodgrass, D.R., Campbell, I., Dawson, A.McL., and Burrells, C. 1981. IgG_1 antibody in milk protects lambs against rotavirus diarrhoea. Vet.Immunol. Immunopathol., 2: 27-33.

Newborn gnotobiotic lambs were fed a diet of diluted evaporated milk supplemented either with normal ewes' milk or with milk obtained from ewes injected parenterally during gestation with rotavirus. Lambs fed 150 ml per day of milk collected 5 days after lambing from normal ewes were susceptible to rotavirus infection and diarrhoea, while lambs fed milk from vaccinated ewes collected either 5 or 12 days after lambing were protected. Analysis of the milk by column chromatography showed the anti-rotavirus activity to be in the fractions containing IgG_1.

INTRODUCTION

Ingestion of antibodies to rotavirus by young animals can prevent rotavirus infection and diarrhoea (Bridger and Woode, 1975; Lecce et al., 1976; Snodgrass and Wells, 1978). This observation led to experiments in which pregnant ewes and cows were vaccinated with inactivated rotavirus in adjuvant, to stimulate them to produce elevated levels of antibody in colostrum and milk after parturition (Wells et al., 1978; Snodgrass et al., 1980). Although milk from vaccinated cows was found to contain high levels of anti-rotavirus IgG_1 antibody for at least 28 days after birth and delayed the onset of rotavirus infections in the suckling calves, it did not prevent disease (Snodgrass et al., 1980).

The experiment reported in this paper was designed to elucidate whether IgG_1 antibodies in milk from vaccinated dams could protect against rotavirus infection, using ewes and lambs as a model ruminant system for cattle. Gnotobiotic lambs were challenged with rotavirus whilst being fed day 5 or day 12 milk from vaccinated

ewes or day 5 milk from control ewes. The milk was pooled on day 5 because of the virtual absence of anti-rotavirus antibody in the 'control' milk (Snodgrass and Wells, 1978) and on day 12, to restrict the protective effect to IgG_1 antibodies in the 'immune' milk.

MATERIALS AND METHODS

Immune and Control Milk

Lamb rotavirus (LRV) was prepared from 10 ml of intestinal contents from the fifth gnotobiotic lamb passage by Arklone (ICI) extraction and banding on a CsCl gradient (Rodger et al., 1975). Four pregnant Greyface (Leicester x Blackface) ewes were injected intramuscularly (I.M.) in the hind legs, 5 to 7 weeks after mating, with 2 ml of an emulsion containing equal parts of LRV and Freund's complete adjuvant (Difco). Although there were no visibly adverse reactions to the adjuvant, the ewes received a second I.M. injection of LRV without adjuvant 2 to 4 weeks before term.

After lambing, the 4 ewes were permitted to suckle their lambs normally until 4 days after birth when their lambs were removed and the ewes milked the following morning. The lambs were returned to the ewes until 11 days after birth when they were removed for a further period of 16 hours and the ewes' milk collected the following day. Milk was obtained from 6 control Greyface ewes in the same manner on the fifth day after lambing.

The milk was obtained by aseptic technique and pools made of day 5 and day 12 'immune' milk and day 5 'control' milk. These pools were sterilized by heating to 56° for 1 h, incubating at 37° for 1 h with 50 µg/ml of Gentamicin (Roussel) and reheating to 56° for 1 h the following day. The pools were tested for sterility, apportioned and stored at -20°.

Passive Protection of Newborn Lambs

Eight gnotobiotic lambs were maintained on evaporated milk from birth. From 24 h to 120 h after birth their diet was supplemented with 50 ml feeds, every 8 h, of either day 5 'control' milk (4 lambs), day 5 'immune' milk (2 lambs) or day 12 'immune' milk (2 lambs). All lambs were infected orally, 28 hours after birth, with 1.5 ml of a 0.22µ filtrate of LRV which had a titre in foetal lamb kidney cells of $10^{4.8}TCID_{50}$/ml and which contained approximately 10^7 particles/ml, as estimated by electron microscopy. Faecal swabs were collected daily from all lambs and examined for rotavirus by countercurrent immunoelectro-osmophoresis (Middleton et al., 1976) using rabbit anti-rotavirus antiserum, or by the ELISA assay. Faecal swabs were also checked for bacterial sterility.

Fractionation of Antibody Activity in 'Immune' Milk

Five ml of whey from each pool of milk was fractionated on a 2.6 x 82cm column of S300 (Pharmacia). The proteins were eluted at 16 ml/h with 0.1M TRIS-HCl:1.0M NaCl buffer pH 8.0 and each 8 ml fraction concentrated to 2 ml by dialysis against Carbowax PEG 20M (Union Carbide) and phosphate buffered saline. Fractions were assayed for the class and concentration of immunoglobulin by single-radial immunodiffusion (Mancini et al., 1965) using sub class specific rabbit anti-sheep immunoglobulin sera (Smith et al., 1975).

Detection of rotavirus and anti-rotavirus antibody by ELISA

An enzyme-linked immunosorbent assay (ELISA) similar to that described by Yolken et al. (1977) was used for the detection of rotavirus. Briefly, microplates were coated with hyperimmune sheep anti-rotavirus IgG. Test faecal extracts were added, followed by rabbit anti-rotavirus IgG conjugated with alkaline phosphatase. Finally phosphatase substrate (Sigma) in diethanolamine buffer was added to the plates which were read 3/4 h later in a multichannel spectrophotometer (Titertek Multiskan, Flow Labs.).

Antibody estimations were performed using a blocking assay. The test was performed as above except that dilutions of test sera were added to the microplates after a positive antigen layer, but before the addition of conjugated anti-rotavirus IgG. The endpoint was taken as the final dilution giving at least a 50% reduction in optical density compared with saline controls.

RESULTS

Passive Protection of Lambs with Immune Milk

Following challenge with rotavirus the lambs receiving the supplementary feeding of day 5 and day 12 'immune' milk showed no signs of clinical disease (Table I). No rotavirus was detected in faeces from any of these 4 lambs.

All 4 lambs fed day 5 'control' milk scoured the day after the oral challenge with rotavirus; the diarrhoea lasting from 1 to 3 days (Table I). One control lamb excreted virus within 24 h, 2 within 48 h and the fourth by 72 h after challenge; virus excretion persisted for 1 to 4 days.

No bacteria were detected in the faeces from the lambs, except for one fed day 12 'immune' milk, which had a persistent streptococcal infection from day 5 of the experiment.

TABLE 1

Passive protection of newborn gnotobiotic lambs against rotavirus diarrhoea

Milk	Lamb No.	Diarrhoea		Virus excretion	
		onset (days p.i.[1])	duration (days)	onset (days p.i.[1])	duration (days)
Day 5 'Immune'	1	No	0	No	0
	2	No	0	No	0
Day 12 'Immune'	3	No	0	No	0
	4	No	0	No	0
Day 5 'Control'	5	1	3	3	1
	6	1	3	1	3
	7	1	1	2	4
	8	1	1	2	3

[1] days P.I. - days post infection with rotavirus.

Analysis of Antibody Activity in Milk

Analysis of the S300 fractions of whey prepared from milk collected 5 days post-partum, indicated that the predominant immunoglobulin present in ewes' milk was IgG_1, with much lower amounts of IgM and IgA. IgG_2 was not detected (< 0.1 g/l). Anti-rotavirus antibody activity, as detected by the blocking ELISA, was concentrated in the fractions containing IgG_1 (Fig. 1a), although there was a slight overlap of activity into the IgA region.

A similar analysis of whey from milk collected 12 days post-partum produced almost identical results, except that IgM could no longer be detected (< 0.1 g/l) and there was no overlap between the fractions containing IgA and IgG_1 (Fig. 1b). In day 12 whey there was no detectable anti-rotavirus activity in the IgA region (Fig. 1b).

Except for an increased 7S peak (fractions 22 to 24), the S300 fractionation of day 5 'control' milk whey produced a similar adsorbance profile to the day 5 'immune' whey (Fig. 1a). Fractions 22 to 24 contained IgG_1 anti-rotavirus antibody. The fractions, which had been concentrated by a factor of 2.5 compared to the original whey, had titres of 1, 2 and 2($\log_2 N$) respectively.

DISCUSSION

In previous work both normal colostrum and hyper-immune serum passively protected gnotobiotic lambs against rotavirus diarrhoea (Snodgrass and Wells, 1978). An attempt to apply those findings to a more natural situation in cattle met with mixed success (Snodgrass et al., 1980). While parenteral vaccination with

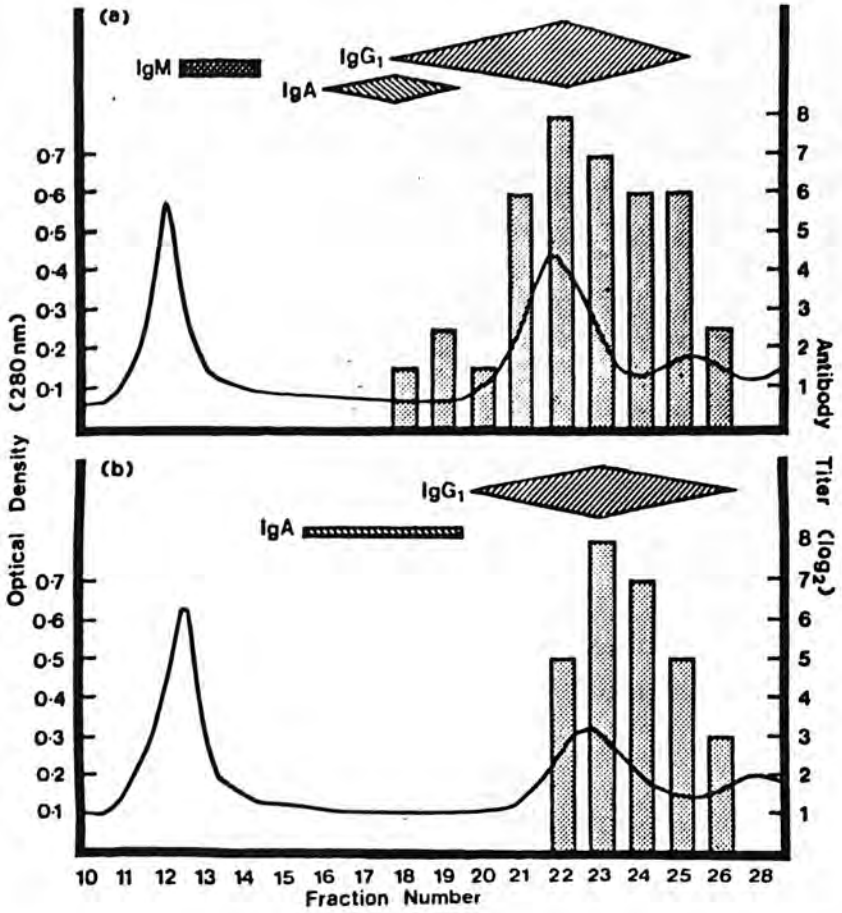

Fig. 1. S300 fractionation of (a) day 5 and (b) day 12 'immune' milk whey. The titre of anti-rotavirus antibody (histogram) and the relative concentration of immunoglobulin (hatched areas) in each fraction is shown against the optical density profile of the fractionated whey.

rotavirus in adjuvant elevated the neutralizing antibody titres in the circulation, colostrum and milk of the dams, the calves succumbed to the rotavirus challenge, albeit with a delayed onset of clinical disease and virus excretion. Analysis of the milk obtained from vaccinated cows 2 to 4 weeks after calving showed that the predominant class of immunoglobulin present and the one having most antibody activity was IgG_1. The occurrence of diarrhea in the calves sucking vaccinated dams meant that either IgG_1 antibodies could not protect the neonate after the effects of colostrum had dissipated, or, as suggested by Snodgrass et al. (1980), that the large rotavirus challenge of approximately 10^{11} virus particles was enough to overcome the passive protection afforded by the milk.

Using the gnotobiotic lamb model, which enables rotavirus diarrhoea to be induced reproducibly and the intake of antibody by the lambs to be controlled, we have been able to demonstrate that the IgG_1 anti-rotavirus antibody in day 12 milk is sufficient to prevent rotavirus infection. Comparing the titres of antibody in 'immune' ovine milk (Snodgrass and Wells, 1978) with those in 'immune' bovine milk (Snodgrass et al., 1980), suggests that equally effective pools of 'immune' ewes' milk could have been obtained at any time during the first month of lactation.

The quantity of 'immune' milk ingested by the lambs (150 ml/day for 3 days) was less than 20% of the total milk consumed during that period and would probably represent a similar proportion of a naturally suckled lamb's intake of milk. A combination of the extremely low titres of antibody in day 5 'control' milk and the quantity of milk fed to the lambs each day, probably accounted for the inability of such milk to protect the lambs against infection.

It is well documented that IgG_1 is the major immunoglobulin in ovine and bovine milk (Lascelles and McDowell, 1974) and that it arises by selective transfer from the circulating pool, rather than by local synthesis in the mammary gland (Lascelles, 1977). Withholding 'immune' milk from lambs for the first 24 h after birth minimized the adsorption of antibodies into the circulation (McCarthy and McDougall, 1953) and ensured that the antibodies were having their effect locally in the gut. It may be that antibodies act by neutralizing the virus, by preventing the virus from attaching to the epithelium, by inhibiting viral replication in epithelial cells or by facilitating virus removal by phagocytic cells. Irrespective of the way antibodies prevent rotavirus diarrhoea in the offspring, this experiment strongly supports the rationale for vaccinating dams to prolong the presence of IgG_1 anti-rotavirus antibodies in the milk. The difference between the protection recorded in this experiment in lambs and the results of the bovine vaccination experiment could be explained by the marked difference in the dose of challenge virus.

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Research in Veterinary Science 1982, 32, 70–73

Diarrhoea in dairy calves reduced by feeding colostrum from cows vaccinated with rotavirus

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Forty-two dairy calves remained with their dams for two days after birth, and then were removed to a calf rearing shed. Calves were allocated to three groups for the next 14 days, and received twice daily either whole milk, whole milk with a 10 per cent supplement of pooled normal bovine colostrum or whole milk with 10 per cent supplement of colostrum from cows vaccinated with rotavirus. A natural outbreak of diarrhoea occurred, affecting 28 of the 42 calves. Feeding immune colostrum delayed the onset of diarrhoea, and reduced its incidence, duration and severity. Live weight gains were consequently improved. The group fed normal colostrum had diarrhoea intermediate in severity between that of control calves and those fed immune colostrum. The aetiology of the diarrhoea was complex, with calves excreting rotavirus, enteropathogenic *Escherichia coli* and cryptosporidia.

ROTAVIRUS, coronavirus, enteropathogenic *Escherichia coli* and cryptosporidia are all potentially significant in the aetiology of calf diarrhoea (Acres et al 1975, Morin et al 1976, Moon et al 1978, de Leeuw et al 1980). The relationship between diarrhoea and continued colostrum feeding is not clear (Foley and Otterby 1978). In addition to the passive immunity transferred from cow to newborn calf by intestinal absorption of intact immunoglobulins (Selman 1972), colostral immunoglobulins can exert a local protective effect in the gut (Logan et al 1974, Snodgrass and Wells 1978). As excess colostrum is generally available on dairy farms, the diet of dairy calves was supplemented with normal colostrum and colostrum from cows vaccinated with rotavirus. The effect of these regimes on an outbreak of diarrhoea in the calves is recorded.

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Materials and methods

Animals

Forty two Friesian, Jersey and crossbred calves of both sexes born in a paddock remained with their dams for 36 to 48 hours. They were then brought in to a calf rearing house and randomly allocated to three treatment groups: normal colostrum (11), immune colostrum (10) or control (21).

For the first two experimental feeds, calves were trained to drink from a rubber nipple and between these feeds were kept in a mixed group pen. They were then placed in individual raised pens with a wire mesh floor in an uncontrolled environment calf house which had not been used for 10 months. Each pen measured 1·22 m by 0·51 m and permitted oral contact between adjacent calves. Calves were randomly allocated to pens, and entered the experiment over a period of 11 days. Each calf remained on experiment for 14 days. The mean initial weight was 31 kg.

Feeding

Calves were fed whole milk twice daily from

the other two dairy herds (68 cows) with prevaccination rotavirus problems, there was a significantly decreased

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1972, Twiehaus and others 1975, Thurber and others 1977). In addition, the rotavirus antibody titres in serum and milk of cows have not been significantly increased after modified live rotavirus vaccination (Myers and Snodgrass 1982, Saif and others 1984, Waltner-Toews and others 1985).

However, experimental studies with inactivated ad-

individual buckets. Those over 25 kg initial weight were offered 4 litres per day; those under 25 kg were offered 3 litres per day. Colostrum supplement was fed to calves by replacing 200 ml of milk with 200 ml of the appropriate colostrum pool at each feed.

Calves with diarrhoea were starved and treated with Vytrate (which contains sodium chloride, glycine, potassium dihydrogen phosphate, anhydrous citric acid, potassium citrate (total 20 g) plus dextrose (44 g) dissolved in 1 litre water per feed (Whitmoyer Laboratories) for four feeds, then given two feeds of half the normal quantity of milk plus an equal quantity of water, after which normal diet was resumed.

Colostrum

A pool of normal colostrum from the first and second milkings after calving was made during spring 1979. The pool was dispensed in 200 ml bottles and stored at −20°C for one year.

The pool of immune colostrum was obtained from first and second milkings from 27 vaccinated cows in spring 1980 and was dispensed and stored at −20°C for one week.

Whey prepared from each of these colostral pools was fractionated on S-300 (Pharmacia) (Snodgrass et al 1980b) to correlate antibody titre with immunoglobulin class.

Vaccination

A calf rotavirus isolate (McNulty et al 1976) was grown in an embryonic rhesus monkey kidney cell line (MA 104) to a titre of $10^{6.6}$ TCID50/ml, inactivated in 0·5 per cent formaldehyde and emulsified in an equal volume of Freund's incomplete adjuvant (Snodgrass et al 1980b). Approximately 10 weeks before calving 2 ml of this emulsion was inoculated intramuscularly at two sites in the neck. A similarly prepared second injection was given four weeks before calving.

Observations on calves

Calves were bled for serum at entry to the trial and were weighed before the morning feed on the first and last days of the trial.

All calves were observed twice daily, with particular attention being paid to the character of the faeces. Faecal fluidity was estimated on a scale of 1 to 5, representing a range of firm to watery faeces, and faecal volume was assigned numerical values of 0 to 2, representing small to large volumes. Faeces were considered diarrhoeic with a fluidity of 4 or 5 or with a medium or large volume of fluidity 3. The daily numerical values of fluidity and volume were added to obtain a 'faecal index' for each calf for the duration of the experiment.

Rectal swabs were taken from individual affected calves on the first and second days of each episode of diarrhoea or on the eighth and 13th days of the trial from calves that remained normal.

Microbiological examination of faeces

Faeces samples were examined by enzyme linked immunosorbent assay (ELISA) for rotavirus (Fahey et al 1981). Nineteen samples were examined by electron microscopy to detect the presence of other enteric viruses.

Three randomly selected colonies of *E coli* isolated from each faecal swab were passaged four times in tryptone soya broth (Oxoid) and grown for 18 hours on Minca-isovitalex agar (Isaacson et al 1978). The presence of K99 pilus antigen was tested for by slide agglutination with a K99 antiserum prepared in rabbits by inoculation of a K99+ mutant of a K12 strain, with subsequent absorption of the serum by the parent K12 strain (Moon et al 1977).

Smears were made from all faecal samples, which were stained by Giemsa's method and examined for the presence of cryptosporidia (Pohlenz et al 1978, Snodgrass et al 1980a).

Examination of serum and whey

Calf serum was tested by the zinc sulphate turbidity test, to determine approximate immunoglobulin levels (McEwan et al 1970).

Whey pools and samples from S-300 whey fractionations were tested for the presence of neutralising antibody to tissue culture adapted calf rotavirus on MA104 cells grown in microtitre plates. Titres were expressed as the reciprocal of the highest dilution giving complete neutralisation.

Antibody titres to K99 in the whey pools were determined by agglutination and haemagglutination assays.

Statistical analysis

Discrete data were analysed by Fisher's exact test of independence (Goulden 1952). All other data were subjected to analysis of variance and means sequentially tested (Snedecor 1956).

Results

Colostrum pools

Whey prepared from the normal colostrum pool had a rotavirus antibody titre of 320. By S-300 fractionation this antibody was found to be mainly in

the other two dairy herds (68 cows) with prevaccination rotavirus problems, there was a significantly decreased

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1972, Twiehaus and others 1975, Thurber and others 1977). In addition, the rotavirus antibody titres in serum and milk of cows have not been significantly increased after modified live rotavirus vaccination (Myers and Snodgrass 1982, Saif and others 1984, Waltner-Toews and others 1985).

However, experimental studies with inactivated ad-

TABLE 1: Effect of colostrum supplements on diarrhoea and weight gain

Treatment group (number in group)	Number died	Number with diarrhoea	Number with two episodes of diarrhoea	Experiment days to onset of diarrhoea	Days with diarrhoea: All calves	Days with diarrhoea: Diarrhoeic calves	Faecal index: All calves	Faecal index: Diarrhoeic calves	Liveweight gain (g per day)†
Control (21)	1	16	8	3·9	2·3	3·1	50·7	56·4	110
Normal colostrum (11)	1	8	1	4·1	1·4	2·0	41·8	45·8	201
Immune colostrum (10)	0	4	0*	6·0	0·5*	1·2*	28·6*	35·8	270*

* Measurement significantly different from control value ($P<0·05$)
† Covariance corrected for initial weight and relative feeding level (milk/kg initial weight)

the IgA and IgG1 regions. The rotavirus antibody titre of the immune colostrum pool was 2560, with the highest titre located in the IgG1 region of the fractions. No antibody to K99 was detected in either colostrum pool.

Zinc sulphate turbidity tests

Serum from 34 of the 42 calves at entry to the trial had normal ZST values equivalent to more than 20 g per litre IgG. Eight calves had zinc sulphate turbidity levels corresponding to approximate IgG concentrations of less than 20 g per litre, indicating insufficient colostrum intake.

Occurrence of diarrhoea

Diarrhoea occurred in 28 of the 42 calves (Table 1), and two calves died within 48 hours of its onset. Five of eight hypogammaglobulinaemic calves became diarrhoeic, a prevalence similar to that in the total population.

Calves fed immune colostrum supplement tended to have a lower incidence of diarrhoea and at a later age than the controls (Table 1). Duration and severity of diarrhoea (faecal index) were significantly reduced in calves in this group.

The incidence of diarrhoea and age at onset were similar in calves receiving normal colostrum supplement and in controls (Table 1). Duration and severity of diarrhoea in calves fed normal colostrum were of intermediate value between the other two groups.

Growth

The liveweight gain data were corrected for relative feeding level, which varied with initial weight and treatment for diarrhoea. Weight gain was significantly improved by feeding immune colostrum (Table 1).

Microbiological observations

Infections with rotavirus, K99+ *E coli* and cryptosporidia were common (Table 2). Dual infections occurred frequently in the control calves, but concurrent infections with all three organisms were detected in only two calves. Examination of 19 faeces samples by electron microscopy did not reveal the presence of enteric viruses other than rotavirus. Salmonella were not isolated from any sample.

Rotavirus infections occurred commonly in first and second diarrhoea episodes in control calves but less commonly in calves receiving either colostrum supplement. K99+ *E coli* were isolated mainly from control calves during the first diarrhoea episode, and from diarrhoeic calves receiving normal colostrum supplement. Cryptosporidia were detected in faeces from diarrhoeic and normal calves in all groups.

Discussion

Vaccination of cows with an inactivated calf rotavirus vaccine increased the colostral rotavirus antibody titres. Storage at −20°C has been shown to preserve nutrient quality of colostrum (Foley and

TABLE 2: Occurrence of enteropathogens in faeces samples

	Control calves: 1st episode of diarrhoea	Control calves: 2nd episode of diarrhoea	Control calves: Normal calves	Normal colostrum supplement: 1st episode of diarrhoea	Normal colostrum supplement: 2nd episode of diarrhoea	Normal colostrum supplement: Normal calves	Immune colostrum supplement: Diarrhoeic calves	Immune colostrum supplement: Normal calves
n	16	8	5	8	1	3	4	6
Rotavirus	6	6	4	2	0	2	1	3
K99+ *E coli*	7	1	2	4	0	0	0	1
Cryptosporidium	4	2	5	4	1	2	4	5

The first detection only of each organism in each individual calf is recorded

Otterby 1978), so the constitution of the two colostrum pools from the same herd in successive years could be expected to be similar except for the rotavirus antibody titres.

Feeding calves the colostrum supplement from cows vaccinated with rotavirus reduced the incidence of diarrhoea, which was also less severe and of shorter duration than in control calves. In addition, the weight gains of the calves fed immune colostrum were increased, and as these calves tended also to grow faster than calves fed normal colostrum, their improved weight gains were probably due to a reduction in diarrhoea as well as to the greater nutritional value of colostrum. These beneficial effects associated with continued feeding of immune colostrum were associated with a reduction in both rotavirus and K99+ *E coli* infections.

The precise aetiological role of each agent in this diarrhoea outbreak is impossible to define. All three enteropathogens were detected in nondiarrhoeic as well as diarrhoeic calves. Colostrum containing high titre rotavirus antibody reduced the incidence of both rotavirus and K99+ *E coli* infections, although no antibody to K99 could be detected. This supports the suggestion that enterotoxigenic *E coli* colonise the intestine less readily in the absence of rotavirus infection (Runnels et al 1980, Snodgrass et al 1981).

Calves in this experiment were exposed naturally to a severe multiple-aetiology outbreak of diarrhoea, and under these field conditions the continued dietary supplementation with colostrum from rotavirus-vaccinated cows had a beneficial effect in reducing the occurrence and severity of diarrhoea and increasing the performance of the calves. Further investigation of this method of preventing diarrhoea in dairy calves is warranted. In particular it may be possible to combine rotavirus vaccination with an *E coli* vaccine that raises K99 colostral antibody titres (Acres et al 1979).

Acknowledgements

We thank Dr K. J. Fahey for colostrum fractionation and Dr T. K. S. Mukkur for K99 haemagglutination inhibition assays. We also thank the staff at the Dairy Research Institute, Ellinbank for care of the calves.

Received for publication March 13, 1981

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INFECTION AND IMMUNITY, Aug. 1982, p. 586–591
0019-9567/82/080586-06$02.00/0

Vol. 37, No. 2

Passive Immunity in Calf Diarrhea: Vaccination with K99 Antigen of Enterotoxigenic *Escherichia coli* and Rotavirus

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Received 23 February 1982/Accepted 27 April 1982

Twenty-four pregnant cows were vaccinated intramuscularly with K99 extract from enterotoxigenic *Escherichia coli* and inactivated rotavirus as follows: six cows were injected with 2 ml of oil-adjuvanted vaccine; six cows were injected with 0.5 ml of oil-adjuvanted vaccine; six cows were injected with 4 ml of aluminum hydroxide-adjuvanted vaccine twice with a four-week interval; and six cows were unvaccinated as controls. Calves born to these cows were challenged with enterotoxigenic *E. coli* at 6 to 18 h after birth. Serum and milk antibodies to K99 and rotavirus in cows vaccinated with either dose of oil vaccine were significantly increased until at least 28 days after calving. In cows vaccinated with alhydrogel vaccine, there was a significant K99 antibody increase in serum and in colostrum but not in milk and a significant rotavirus antibody increase only in colostrum. Five of six calves born to unvaccinated cows developed enterotoxic colibacillosis after challenge, and all excreted the challenge strain of enterotoxigenic *E. coli*. None of the 18 calves in the three vaccinated groups developed clinical colibacillosis, and fecal excretion of the challenge organism was reduced. A combined enterotoxigenic *E. coli*-rotavirus vaccine may prove useful in preventing some outbreaks of calf diarrhea.

Although many infectious agents have been implicated in the etiology of diarrhea in young calves, four microorganisms stand out as being of widespread occurrence and proven enteropathogenicity: rotavirus, coronavirus, enterotoxigenic *Escherichia coli* (ETEC), and cryptosporidia (2, 8, 10, 11, 23).

Control of diarrhea has been attempted with a live attenuated rotavirus-coronavirus vaccine for oral inoculation of newborn calves (Scourvax-II; Norden Laboratories) but has not been proven effective in blind field trials (3, 9). Control can also be attempted through dam vaccination to elevate the titers of specific antibody ingested by the calf in colostrum and milk. Such an approach has been used successfully with both bacterins and with K99 pili from ETEC (1, 14, 15), although live attenuated rotavirus-coronavirus vaccination of pregnant cows (Calf Guard; Norden Laboratories) does not significantly raise milk antibody titers (L. L. Myers and D. R. Snodgrass, J. Am. Vet. Med. Assoc., in press). The use of inactivated adjuvanted rotavirus vaccine results in greatly increased colostrum and milk antibody production (20, 21).

The objectives of this study were to combine K99 pili from ETEC with a rotavirus vaccine for pregnant cows and to assess the efficacy by serology and by challenging newborn calves with ETEC.

MATERIALS AND METHODS

Animals. A total of 24 pregnant hill suckler cows from 7 to 15 years of age were allocated to treatment groups at 4 to 10 weeks before calving. Six cows were vaccinated once with 2 ml of oil-adjuvanted vaccine by deep intramuscular injection in the neck; six cows were similarly vaccinated once with 0.5 ml of the same oil-adjuvanted vaccine; six cows were vaccinated twice with a 4-week interval with 4 ml of aluminum hydroxide-adjuvanted vaccine by deep intramuscular injection in the neck; and six cows were not vaccinated. Four of the cows allocated to the control group had been vaccinated with rotavirus-coronavirus vaccine in their previous pregnancy and were included as controls only for the ETEC component.

The cows were housed before calving for the duration of the experiment. Calves were challenged with ETEC between 6 and 18 h after birth. After challenge, cow-calf pairs from the different treatment groups were kept apart for at least 3 days to prevent cross-suckling and then were moved to a pen separate from that of the uncalved cows.

***E. coli* cultures.** The following serotypes of *E. coli* were used: O101:K-:K99 (designated ETEC 1); O9:K30(B):K99 (designated ETEC 2); O9:K35(A):K99 (designated ETEC 3); and O8:K85ab:K99 (designated ETEC 4).

Vaccines. For the preparation of alhydrogel-adjuvanted vaccine, the K99 component was derived from

the other two dairy herds (68 cows) with prevaccination rotavirus problems, there was a significantly decreased

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1972, Twiehaus and others 1975, Thurber and others 1977). In addition, the rotavirus antibody titres in serum and milk of cows have not been significantly increased after modified live rotavirus vaccination (Myers and Snodgrass 1982, Saif and others 1984, Waltner-Toews and others 1985).

However, experimental studies with inactivated ad-

TABLE 1. Mean K99 antibody titers (measured by ELISA) in cow serum, cow whey, and calf serum after K99-rotavirus vaccination

Vaccine[a]	Titer (log_{10}) in cow serum			Titer (log_{10}) in whey at following day after calving:						Titer (log_{10}) in calf serum at following day of age	
	Before vaccination	4 Wk after vaccination	At calving	1	3	7	14	28	90	3	28
None (control)	1.9	1.8	1.8	2.0	1.7	1.7	1.7	1.7	1.7	1.8	3.2
Oil (2 ml)	1.5	4.2[b]	4.5[b]	4.3[b]	4.1[b]	3.6[b]	3.5[b]	3.1[b]	2.9[b]	4.3[b]	4.0[b]
Oil (0.5 ml)	2.0	3.9[b]	4.0[b]	4.3[c]	3.9[b]	3.3[b]	2.8[b]	2.3[c]	2.6[b]	4.2[b]	3.6
Alhydrogel	1.7	2.1	3.1[b]	3.1[b]	2.6[b]	1.9	1.9	1.7	1.7	2.3[c]	2.6

[a] There were six cows in each vaccine group.
[b] Differs significantly from control ($P < 0.01$).
[c] Differs significantly from control ($P < 0.05$).

the culture supernatant of ETEC 1, which was grown in a synthetic medium containing selected amino acids, trace salts, and lactose and was buffered with phosphates to pH 7.5. After 8 to 10 h of incubation at 37°C in an aerated vessel, the culture was inactivated in situ at 60°C for 30 min. The cells were separated aseptically from the supernatant by centrifugation. After measurement of K99 antigen in the sterile culture supernatant, 20% alhydrogel was added.

Tissue culture-adapted calf rotavirus was prepared and inactivated as previously described (20). The rotavirus and K99 components were blended aseptically with alhydrogel in the proportions 1:1:8 so that each milliliter of the combined vaccine contained 15 U of K99 and $10^{5.4}$ 50% tissue culture infective doses of rotavirus before inactivation.

For the preparation of oil-adjuvanted vaccine, the K99 component was derived from ETEC 1 grown on 5% horse blood agar at 37°C for 18 h. The growth was harvested in sterile saline and concentrated by centrifugation so that it contained 2.50×10^{11} cells per ml. Portions (20 ml each) of the concentrate were homogenized in a Silverson homogenizer for 4 min at 0°C. The cells were separated from the supernatant at 20,000 × *g* for 30 min; then 0.1% Merthiolate was added, and the preparation was heated at 60°C for 30 min. The sterility of this crude K99 extract was checked, and its K99 content was measured before use.

Rotavirus and K99 were mixed with 0.2% Tween 80, and this aqueous phase was emulsified with 2 volumes of oil adjuvant (90% Marcol 52 [Esso], 10% Arlacel A [Sandria Chemicals]). Each milliliter of vaccine contained 60 U of K99 and $10^{5.2}$ 50% tissue culture infective doses of rotavirus before inactivation.

ETEC challenge. Strain B44 (ETEC 2) was grown in Trypticase soy broth (BBL Microbiology Systems) for 8 h and then on Minca-IsoVitaleX (BBL) (6) agar for 18 h at 37°C. The bacteria were suspended in phosphate-buffered saline with 10% dimethyl sulfoxide and stored in 10-ml aliquots at −70°C.

To inoculate each calf, one 10-ml aliquot was thawed and given orally by syringe. The mean inoculum titer was 4.1×10^{10} colony-forming units per 10 ml (range, 3.0×10^{10} to 6.8×10^{10} colony-forming units per 10 ml). No decrease in the inoculum titer occurred over the 2-month experimental period. The enteropathogenicity of the stored organisms was confirmed periodically by slide agglutination for K99 and the infant mouse test for heat-stable toxin production (4).

K99 serology. Antibodies to K99 were assayed by enzyme-linked immunosorbent assay (ELISA) or passive hemagglutination (PHA). The ELISA utilized rabbit anti-K99 immunoglobulin G (kindly supplied by W. H. Jansen) as capture antibody, followed successively by K99 antigen, test serum or whey, and rabbit anti-bovine immunoglobulin G (Miles Laboratories, Inc.) conjugated with alkaline phosphatase. The final phosphatase substrate (Sigma Chemical Co.) reaction was read at 405 nm after 2 h at room temperature. In each test, doubling dilutions of a standard bovine anti-K99 serum were included. The titers of the test samples are expressed in relation to a calibration curve calculated from the standards.

In the PHA assay, pyruvic acid-stabilized sheep erythrocytes (7) were sensitized with K99 antigen from ETEC 4. A suspension of erythrocytes in 0.1 M acetate buffer (pH 4.5) was coated to saturation with K99 derived by the method of Morris et al. (12), washed five times in phosphate-buffered saline (pH 7.5), and resuspended to 1% (vol/vol). Test sera and whey samples were adsorbed with an equal volume of packed unsensitized erythrocytes for 18 h at 4°C to remove nonspecific hemagglutinins. Serial doubling dilutions of serum or whey samples in 0.3% Formol saline were prepared in microtiter plates, and an equal volume (0.025 ml) of sensitized erythrocytes was added. The agglutination pattern was read after 18 h of incubation at 37°C. All of the samples were tested on one occasion, although repeat tests on selected samples yielded the same titers.

Rotavirus serology. Serum samples, whey from colostrum samples, and whey from milk samples were tested for the presence of neutralizing antibody to tissue culture-adapted calf rotavirus on bovine embryo kidney cells or MA104 cells grown in microtiter plates.

Titration of K99 antigen. The titration method used is based on the capacity of the K99 antigen to adsorb K99 antibodies from a standard antiserum of known titer, which is then titrated for residual antibody by PHA. This method is based on an in vitro assay designed for quantitating K88 antigens of *E. coli* (16). The results are expressed as agglutinin absorbing units. The standard antiserum used was produced in a pig vaccinated with a sterile culture supernatant of ETEC 3. The serum was adsorbed to remove all detectable O9 and K35(A) agglutinins.

Toxicity of K99 components in combined vaccines. The toxicity of the K99 vaccine preparations was examined by injecting groups of 10 mice intraperitoneally with 15 U of alhydrogel-adsorbed K99 and 180 U of crude K99 extract.

Feces examination. Calf feces samples were examined for rotavirus by ELISA (5) and for cryptosporidia by examination of Giemsa-stained fecal smears (19). Samples were cultured on Minca-IsoVitaleX (6) and MacConkey agars overnight aerobically at 37°C. Five colonies grown on Minca-IsoVitaleX agar were tested for K99 by slide agglutination with rabbit antiserum to strain K12:K99 adsorbed with the K12 strain.

At intervals, fecal swabs from all calves were tested for the presence of *Salmonella* spp. after overnight enrichment in Selenite broth and for *Campylobacter* spp. by growth on 5% sheep blood agar plus Skirrows antibiotic supplement (Oxoid Ltd.) under microaerophilic conditions at 37°C.

Observations. Each cow was bled for serum at vaccination, 4 weeks after vaccination, at parturition, and 28 days after parturition. Colostrum and milk samples were collected at 1, 3, 7, 14, 28, and 90 days after calving. The calves were bled for serum at 3 and 28 days of age. Feces samples were collected from the calves daily for 6 days. A sample of these feces was taken for microbiological examination, and the remainder was dried to constant weight for dry-matter estimation. The calves were weighed at 1, 2, and 3 days of age. All calves were examined clinically at least once a day for 6 days and assigned a clinical score on a subjective scale similar to that used by Myers (14): (i) normal, feces firm; (ii) transient diarrhea within 24 h of inoculation, lasting only a few hours; (iii) severe watery diarrhea, calf becoming dehydrated and dull; (iv) severe watery diarrhea, calf too weak to stand, with death ensuing.

RESULTS

K99 immunological response measured by ELISA. Six cows had low preexisting serum antibody (mean titer, 158), whereas all other cows were negative (titer < 100). All vaccine regimes significantly increased serum antibody titers ($P < 0.001$) (Table 1), although oil vaccines produced significantly higher titers at calving (18,900 and 8,830 for 2- and 0.5-ml doses, respectively) than did the alhydrogel vaccine (1,190) ($P < 0.001$).

Both 2- and 0.5-ml doses of oil vaccine produced very high titers in colostral whey (32,100 and 19,800, respectively) compared with controls (108) ($P < 0.001$). These colostral antibody levels declined slowly throughout the 28-day observation period, but remained significantly higher than in the controls (2 ml of oil, $P < 0.001$; 0.5 ml of oil, $P < 0.05$). Antibody titers in colostral whey from the cows vaccinated with alhydrogel vaccine were also increased (1,140, $P < 0.001$), but by 7 days after calving, antibody was no longer detectable in this group.

The serum antibody titers in 3-day-old calves reflected the colostral antibody titers of their dams. By 28 days of age, the calves born to unvaccinated cows had developed high serum antibody titers to K99 as a result of ETEC infection.

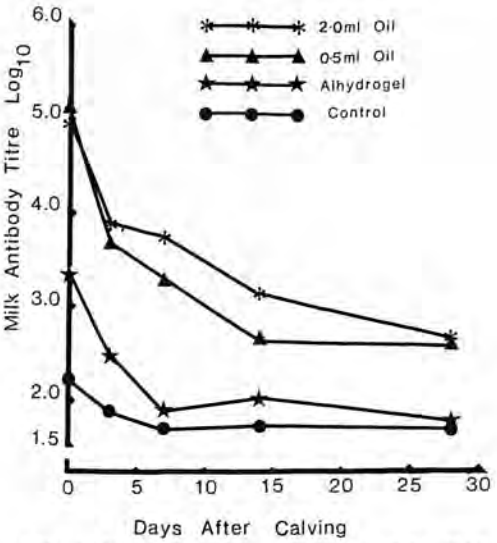

FIG. 1. K99 antibody titers determined by PHA ($\log_{10}$) in colostrum and milk of cows during the first 28 days of lactation.

K99 immunological response measured by PHA. The assay of sera and wheys by PHA confirmed the results obtained by ELISA (Fig. 1). In addition, the cow sera collected 28 days after calving were tested, and K99 antibody titers were found to be still significantly raised in all vaccinated cows.

Rotavirus immunological response. The results from the four control cows which had been vaccinated in a previous pregnancy are excluded from these results, and data from seven extra control cows from the same farm not otherwise included in the experiment are incorporated. All cows had prevaccination serum antibody to rotavirus. The serum and milk antibody responses of cows to both doses of oil-adjuvanted vaccine were significant (Table 2).

Cows vaccinated with alhydrogel-adjuvanted vaccine had raised antibody titers in serum and milk, but only colostral antibody titers were significantly higher than in control cows.

Response of calves to ETEC challenge. After ETEC challenge, five of six calves from control cows developed acute enteric disease (disease rating iii or iv) characterized by profuse watery diarrhea, dehydration, and dullness (Table 3). The mean body weight loss of 5.7% and the mean minimum fecal dry matter of 8.4% confirmed the severity of the disease. One calf died 2 days after challenge.

The clinical responses of calves in all three

the other two dairy herds (68 cows) with prevaccination rotavirus problems, there was a significantly decreased

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However, experimental studies with inactivated ad-

TABLE 2. Mean rotavirus antibody titers (measured by the neutralization test) in cow serum and whey after K99-rotavirus vaccination

Vaccine	Titer (log_{10}) in cow serum			Titer (log_{10}) in whey at following day after calving:				
	Before vaccination	4 Wk after vaccination	At calving	1	3	7	14	28
None (control)	2.51	2.66	2.36	2.81	2.05	2.02	1.83	1.70
Oil (2 ml)	2.66	3.19	3.46[b]	4.06[a]	3.51[b]	2.86[a]	2.51	2.26[a]
Oil (0.5 ml)	2.81	3.71[a]	3.65[b]	3.96[a]	3.46[b]	2.91[a]	2.41	2.43[a]
Alhydrogel	2.66	3.33	3.29	3.51[b]	2.81	2.36	2.15	2.02

[a] Differs significantly from control value ($P < 0.01$).
[b] Differs significantly from control value ($P < 0.05$).

vaccinated groups were similar. A mild transient diarrhea unaccompanied by systemic disturbance was observed in half of the calves (disease rating ii). All calves gained weight, and fecal dry matter did not fall below 16% in any individual.

Microbiological examination of feces. A serological examination of fecal coliforms was performed for 6 days after ETEC challenge. In control calves, 86% of *E. coli* could be identified as ETEC (Table 4). The proportion of B44 excreted in the feces of calves from vaccinated cows was reduced. In only one calf was there no detectable B44 excretion. Rotavirus was detected in the feces of one control calf with diarrhea and in three clinically normal calves born to vaccinated cows. Cryptosporidial oocysts were not observed in the feces of any calf.

Vaccine toxicity. No toxic effects of K99, used in the vaccines, were detected in mice, and all vaccinated cows remained clinically normal.

DISCUSSION

Calves sucking dams vaccinated with K99 pili in any of the schedules used were protected against the clinical effects of challenge with ETEC, whereas the same challenge produced severe enterotoxic colibacillosis in calves from control cows. In addition, excretion of the challenge strain was significantly reduced in calves from vaccinated cows. Although the oil vaccines produced a much higher immunological response than the alhydrogel vaccine, the degree of protection under these experimental conditions was equally satisfactory.

Most natural ETEC infections occur in calves 1 to 2 days old (2, 10), and increased antibody titers in colostrum alone should be protective in these cases. However, there is experimental evidence that initial rotavirus infection can facilitate ETEC colonization in calves up to 1 week old (17, 20a), and such dual infection of older calves has been reported to occur naturally (10). For this reason, it is desirable to stimulate production of K99 antibody in milk as well as in colostrum, and the oil-adjuvanted vaccine formulation was more effective in this regard than the alhydrogel vaccine. The mean K99 ELISA titers of 7-day milk of cows vaccinated with either dose of the oil-adjuvanted vaccine were higher than those in colostrum of cows which received the alhydrogel-adjuvanted vaccine. As calves of the latter group of cows were resistant to experimental challenge, it is reasonable to suggest that cows vaccinated with oil-based K99 vaccine would confer protection to their calves for at least 7 days and probably longer.

With the methods of vaccine production used with strain B41 (O101:K-:K99) it is likely that antigens other than K99 were present, in particular the O101 somatic antigen and an anionic adhesin (13). However, cross-protection against

TABLE 3. Clinical results of ETEC challenge

Vaccine	No. of calves given the following disease rating:[a]				Wt at 48 h/wt at birth (%)[b]	Minimum fecal dry matter (%)[b,c]
	i	ii	iii	iv		
None (control)	0	1	4	1	94.3 ± 3.6	8.4 ± 2.1
Oil (2 ml)	3	3	0	0	107.5 ± 1.5[d]	28.7 ± 3.2[d]
Oil (0.5 ml)	3	3	0	0	106.7 ± 1.6[d]	24.9 ± 2.9[d]
Alhydrogel	3	3	0	0	103.3 ± 1.5[e]	22.6 ± 1.8[d]

[a] See text for definitions of disease ratings.
[b] Mean ± standard error.
[c] Minimum recorded for each calf over the 4 days after ETEC challenge.
[d] Differs significantly from control ($P < 0.01$).
[e] Differs significantly from control ($P < 0.05$).

TABLE 4. Fecal excretion of ETEC after challenge

Vaccine	Colonies agglutinated (%)[a]
None (control)	86
Oil (2 ml)	49[b]
Oil (0.5 ml)	16[c]
Alhydrogel	32[c]

[a] Mean number of colonies agglutinated by K99 antiserum as a percentage of the total number of colonies tested over the 6 days after challenge.
[b] Differs significantly from control ($P < 0.05$).
[c] Differs significantly from control ($P < 0.01$).

the challenge strain O9:K30:K99 suggests that effective protection was produced in this case by K99 antibodies, even against a strain which may possess colonization properties through K30 (18).

These results confirm those of others who have found that antibody to K99 protects against virulent ETEC challenge (1, 15). However, the incorporation of a rotavirus vaccine greatly increases the value of the immunization regime. No rotavirus challenge was included, but the rotavirus serological response produced in this experiment by the oil-adjuvanted vaccines was consistent with that produced previously, which has been shown to confer substantial protection against rotavirus infection (5, 20, 21). Thus, there was no evidence that the inclusion of K99 interfered with the maternal response to rotavirus vaccination.

An effective bivalent vaccine against ETEC and rotavirus would not prevent all calf diarrhea outbreaks. In particular, disease due to calf coronavirus would continue, as has been found with a previous rotavirus vaccine (22), and cryptosporidiosis would continue to be present. However, the use of such a vaccine could be expected to lead to a useful reduction in morbidity and mortality from diarrhea in young suckled calves and potentially in dairy calves also if the duration of colostrum feeding was prolonged (21).

ACKNOWLEDGMENTS

We thank the Hill Farming Research Organisation for their cooperation.

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However, experimental studies with inactivated ad-

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Evaluation of a combined rotavirus and enterotoxigenic *Escherichia coli* vaccine in cattle

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Veterinary Record (1986) **119**, 39-43

A vaccine of rotavirus and K99 antigen from enterotoxigenic *Escherichia coli* was emulsified in oil adjuvant and administered intramuscularly to pregnant cows. Calves born to and reared on vaccinated dams were protected against experimental rotavirus infection at five days old when compared with calves from unvaccinated control cows. Field trials of the vaccine were carried out in 40 commerical herds, in which half the cows in each herd were selected at random for vaccination and half were left unvaccinated. In 31 herds (2641 cows) there was no significant diarrhoea problem (less than 10 per cent morbidity); these herds were excluded from further analysis. The nine remaining herds did experience a calf diarrhoea problem of greater than 10 per cent morbidity, but on four farms the disease was associated with cryptosporidiosis and on a fifth no enteropathogens were detected; these five farms (461 cows) were also excluded from further analysis. Of the remaining four herds, two beef suckler herds (105 cows) had concurrent rotavirus and cryptosporidial infections, and vaccination was associated with a decreased excretion of rotavirus but not with a decreased incidence of diarrhoea. In the other two dairy herds (68 cows) with prevaccination rotavirus problems, there was a significantly decreased incidence of diarrhoea in calves born to vaccinated cows. No natural field challenge of enterotoxigenic *E coli* was encountered on any of the trial farms.

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SURVEYS in Europe and North America suggest that rotavirus and enterotoxigenic (K99+) *Escherichia coli* play a prominent role in the aetiology of the neonatal calf diarrhoea syndrome (Morin and others 1976, Acres and others 1977, Moon and others 1978, de Leeuw and others 1980a, Tzipori 1981, Bulgin and others 1982, Snodgrass and others 1986). Although effective vaccination of the dam against enterotoxigenic *E coli* has been achieved experimentally (Acres and others 1979, Nagy 1980, Myers 1982, Snodgrass and others 1982a), the vaccines have not been adequately evaluated in the field. Rotavirus vaccines based on inoculation of the pregnant cow with modified live virus have not been shown to be of benefit in within-herd trials where only a proportion of the herd was vaccinated (Acres and Radostits 1976, de Leeuw and others 1980b, Waltner-Toews and others 1985). The only suggestions of efficacy have been made when periods during which vaccine has been used in a herd have been compared with historical periods without vaccine (Mebus and others 1972, Twiehaus and others 1975, Thurber and others 1977). In addition, the rotavirus antibody titres in serum and milk of cows have not been significantly increased after modified live rotavirus vaccination (Myers and Snodgrass 1982, Saif and others 1984, Waltner-Toews and others 1985).

However, experimental studies with inactivated ad-

juvanted rotavirus vaccines have consistently demonstrated increased serum antibody titres (Snodgrass and others 1980, Hess and others 1982, Dauvergne and others 1983, Castrucci and others 1984, Saif and others 1984). It has been shown experimentally that the IgG1 antibody present in milk after vaccination of the dam can be protective (Fahey and others 1981, Saif and others 1983) and a within-herd vaccine trial showed good protection against naturally occurring rotavirus diarrhoea in dairy calves (Snodgrass and others 1982b).

In this study an inactivated rotavirus vaccine was combined with K99 extract from enterotoxigenic *E coli* in an oil adjuvant and administered to pregnant cows. The effect of this vaccine on the subsequent development of rotavirus infection and diarrhoea in their calves in experimental trials and in within-herd field trials is recorded.

Materials and methods

Vaccine

The rotavirus component of the vaccine was the UK strain (Bridger and Woode 1975) grown in MA104 cells. The virus was inactivated with formalin, mixed with K99 antigen derived from the supernatant fluid of an enterotoxigenic *E coli* culture grown in a synthetic medium and emulsified in an oil adjuvant as described by Snodgrass and others (1982a).

Experimental challenge

Ten pregnant Hereford cross Friesian or shorthorn cross Galloway heifers received a single 1 ml dose of vaccine by the intramuscular route four to 14 weeks before calving. Six unvaccinated heifers served as controls.

The cows and calves were housed for the duration of the experiment and the calves were reared on their mothers. On the fifth day of life, all the calves were infected orally with 1 ml of a 50 per cent homogenate in saline of faeces from the first gnotobiotic calf passage of a field rotavirus strain. This rotavirus was shown to contain only a single electrophoretype on analysis of double-stranded RNA on polyacrylamide gels (Herring and others 1982). Its serotype was determined (Snodgrass and others 1984) to be type 6 (Hoshino and others 1984) to which the vaccine strain UK also belongs. The calves were examined daily for evidence of diarrhoea and daily faeces samples were examined for rotavirus, coronavirus, cryptosporidium, enterotoxigenic *E coli* (Snodgrass and others 1986). The cows were bled for serum collection before vaccination and at calving, and colostrum and milk samples were collected at intervals up to 12 days after calving. Rotavirus neutralising antibody and K99 ELISA antibody estimations were performed as described by Snodgrass and others (1982a).

Field trials

Trials were carried out on 34 beef suckler herds and six dairy herds, involving a total of 3275 cows. These 40 farms were selected because of the recurrent nature of their calf diarrhoea problem over many years. In beef herds with a seasonal pattern of calving, vaccination was timed for one month before the onset of calving. In dairy herds with more extended calving periods, groups of cows were vaccinated either one month before calving or at drying-off.

Approximately half the cows in each calving group were left unvaccinated. These cows were selected randomly on the basis of alternate presentation in the handling facilities. All the cows were individually identifiable. On one farm only, in the year following a successful within-herd trial, sequential periods of vaccine usage were alternated with periods during which no cows were vaccinated.

The management of the beef herds was not altered by participation in the trial, each cow suckling its own calf. However in the dairy herds it was important to ensure the continued ingestion of antibody by each calf. It was therefore recommended that the calves should receive their dam's colostrum for the first feed, and that subsequently they should be fed from a pool of first to fourth day colostrum taken from all the vaccinated cows. This pool was stored at ambient temperatures and was replaced every few days by a fresh pool. In this way sufficient colostrum was usually available to feed the calves for 14 days although occasionally the colostrum was diluted with up to 50 per cent of normal milk. The same regime was adopted to feed pooled control colostrum to the calves born to unvaccinated cows. After 14 days the calves were reared on the diet in normal use on that farm.

The trial was monitored by regular farm visits. Routine detection of diarrhoea was carried out by the farmer, diarrhoea being defined as faeces so loose or watery that the animal required treatment. On some farms faeces samples were taken from the calves before treatment and submitted to the laboratory by post. All the samples were examined for rotavirus, coronavirus, cryptosporidium, enterotoxigenic *E coli* and *Salmonella* species as described by Snodgrass and others (1986).

The cows on some farms were bled for serum collection at vaccination and up to seven months after vaccination. Rotavirus neutralising antibody titres were estimated as previously described (Snodgrass and others 1982a).

Statistical methods

Proportional comparisons were carried out using χ^2 or Fisher's exact test as appropriate. Other comparisons were made using Student's t test.

TABLE 1: Rotavirus excretion in faeces from challenged calves

	Controls	Vaccinates	Significance (P)
Number of calves excreting rotavirus/number in group	6/6	4/10	0·03
Mean prepatent period (days, sem)	2·5, 0·4	6·2, 0·8	<0·01
Mean duration of virus excretion (days, sem)	3·7, 0·4	0·9, 0·4	<0·01

Results

Experimental challenge

Vaccinated cows showed significantly increased antibody titres to both rotavirus and K99 in serum and colostral and milk whey until at least 12 days after calving. The titres were similar to those described in previous experiments (Snodgrass and others 1982a).

Calves born to vaccinated cows were protected against the rotavirus challenge infection, with virus excretion being detected in four of the 10 calves in the vaccinated group and in all six of the calves in the control group ($P=0·03$, Table 1). Control calves excreted rotavirus sooner and for a longer period ($P<0·01$, Table 1). Representative faecal samples from 14 calves were examined by polyacrylamide gel electrophoresis. In all cases the electrophoretic pattern of the dsRNA segments was shown to be the same as that of the challenge strain, suggesting that accidental infection with a field strain was unlikely to have occurred. Virus was detected in the same faeces samples by polyacrylamide gel electrophoresis and ELISA, thus eliminating the possibility of false negative results through failure to detect antigen-antibody complexes by

TABLE 2: Detection of rotavirus and cryptosporidium in faeces from diarrhoeic calves in two beef suckler herds

Farm	Vaccinated group n	Number excreting rotavirus	Number excreting cryptosporidium	Control group n	Number excreting rotavirus	Number excreting cryptosporidium
SL	9	0*	6	9	4	4
MC	9	1*	5	13	7	7

* Significantly different from control group ($P<0.05$)

TABLE 3: Occurrence of diarrhoea in two dairy herds

Farm	Vaccinated group Number with diarrhoea/ number in group	Control group Number with diarrhoea/ number in group	Significance (P)
WG	2/11	6/7	<0.01
DL	2/14	18/22	<0.01

ELISA. No coronavirus or enterotoxigenic *E coli* were detected in any calf, but cryptosporidium was observed in faecal smears from six calves.

Diarrhoea occurred in five of 10 and five of six calves in the vaccinated and control groups, respectively. The mean incubation periods were five-and-a-half and three days, and the mean durations of diarrhoea were 1·6 and 2·7 days in the vaccinated and control groups, respectively. None of these differences was significant, but because of the concurrent presence of cryptosporidium it was difficult to associate the occurrence of diarrhoea with rotavirus infection. Faeces samples from all the calves were examined daily and a rotavirus aetiology was diagnosed if rotavirus was detected in the faeces on the day of onset of diarrhoea. On this basis rotavirus diarrhoea occurred in three of six control calves and one of 10 vaccinated calves.

Field trial

Post vaccination sera from 47 cows showed significantly increased neutralising antibody titres to several rotavirus serotypes (Snodgrass and others 1984).

Following vaccination of half the cows there was no significant diarrhoea problem (less than 10 per cent morbidity) in 31 of the 40 herds (2641 cows). However, diarrhoea affecting more than 10 per cent of the calves occurred in the remaining nine farms (641 cows). Using the criteria described by Snodgrass and others (1986) the principal aetiological agents in these nine outbreaks were rotavirus in two, rotavirus and cryptosporidium in two and cryptosporidium in four; on one farm no agent was diagnosed. There was no evidence of enterotoxigenic *E coli* infection on any farm.

In the four herds in which rotavirus either with or without concurrent cryptosporidiosis was diagnosed there was statistical evidence in the vaccinated groups of protection against both virus infection and diarrhoea. In beef suckler herds SL and MC rotavirus was detected in four of nine and seven of 13 control calves, respectively, but in none of nine and one of nine calves in the respective vaccinated groups ($P<0.05$, Table 2). However, because of the concurrent presence of cryptosporidiosis in the control and vaccinated calves on both farms (Table 2) the incidence of diarrhoea was not affected. Without knowing the results of the microbiological examination of faeces, both farmers had the subjective impression that the diarrhoea was much milder in the vaccinated calves. In dairy herds DL and WG diarrhoea occurred in 18 of 22 and six of seven control calves, respectively, but in only two of 14 and two of 11 calves in the respective vaccinated groups ($P<0.05$, Table 3). Both these herds had well characterised rotavirus diarrhoea problems before the start of the trial (rotavirus was detected in 12 of 14 and 40 of 43 scouring calves in herds DL and WG, respectively). The scouring that continued during the trial was also caused by rotavirus on farm WG (rotavirus in five of five faeces samples) but no faeces samples were received from farm DL during the trial.

During the following calving season from August to January on farm WG, comparisons were made between periods when all the cows were vaccinated and periods during which no vaccination was practised. The results were as follows: during period 1 the first 32 cows to calve in the autumn were all unvaccinated; no diarrhoea occurred in the first 18 calves, but the next 14 all scoured. During period 2 three vaccinated and 10 control cows calved but colostrum from the vaccinated cows was fed to all 13 calves by the farmer; no diarrhoea occurred. During period 3 all 43 cows were vaccinated and no diarrhoea occurred in their calves. During period 4 the last 23 cows to calve were not vaccinated; no scouring occurred in the calves from the first 14 but eight of the last nine calves contracted diarrhoea.

Discussion

Calves that continue to be fed colostrum from cows vaccinated with inactivated adjuvanted rotavirus vaccines can be protected against experimental (Saif and others 1983, Castrucci and others 1984) and naturally occurring (Snodgrass and others 1982b) rotavirus infection and diarrhoea. The use of such vaccines which depend upon colostral immunity, is appropriate in dairy herds and the successful within-herd trials conducted on two dairy herds in this study confirm the value of the rotavirus vaccine. Rotavirus vaccination of cows in dairy herds can only be successful if colostrum continues to be fed to the calves throughout their period of exposure to rotavirus, thus ensuring that rotavirus antibody is continuously present in the lumen of the intestine (Snodgrass and Wells 1978). In these trials colostrum feeding until two weeks old was found to be both practical for the farmer and sufficient to control rotavirus diarrhoea.

In beef suckler herds, the continued presence of antibody in the intestine to protect the calf can only be ensured if vaccination stimulates the production of rotavirus antibody in post colostral milk. Rotavirus antibodies in milk are predominantly of the IgG1 isotype and are excreted in the milk of vaccinated cows for at least one month after calving (Snodgrass and others 1980, Hess and others 1982, Saif and others 1984). Such antibodies have been shown to be protective experimentally in lambs (Fahey and others 1981) but there are no reports of such protection in calves. The results of both the rotavirus challenge experiment and the within-herd vaccine trials on the two beef suckler farms where rotavirus was a problem indicate that rotavirus vaccination can indeed be beneficial in suckler herds.

A similar rotavirus vaccine and challenge experiment carried out previously showed that vaccinated cows protected their calves only to the extent of delaying rotavirus infection (Snodgrass and others 1980). It is now known that the rotavirus challenge used in those experiments contained more than one serotype (Bridger and Brown 1984, Bridger personal communication). In addition a very large challenge dose was used which may have overwhelmed the protective antibody present. Rather than conduct stochiometric experiments to define the precise relationship between virus and antibody, it was felt to be more appropriate to test the protection afforded by the vaccine against unquantified field rotavirus challenges. The success of the trials justified this approach.

The difficulties of conducting field trials with vaccines against components of a multiple aetiology syndrome were well illustrated in this study. There was no control of the enteropathogens present and five farms experienced calf diarrhoea problems caused by other enteropathogens (cryptosporidia in four and an undiagnosed agent in a fifth). In addition calves on two of the farms with a rotavirus problem were affected concurrently with cryptosporidiosis, which

partially obscured the clinical benefits of vaccination. Also, no field challenge of the enterotoxigenic *E coli* component of the vaccine was encountered. Finally, no serious diarrhoea problems occurred in the great majority of herds. It is tempting to speculate that this was associated in some herds with the fact that vaccinating 50 per cent of the cows increased herd immunity. Indeed it has been suggested that within-herd trials are inappropriate because of herd immunity mechanisms (Thurber and others 1977). However, the dangers of relying on historical comparisons are obvious and have been clearly demonstrated (Waltner-Toews and others 1985). The validity of the 50 per cent within-herd trial was confirmed in these studies and such trials have the great advantage of producing statistically analysable data.

The studies described in this paper provide evidence of the efficacy of the rotavirus component of the vaccine under both experimental and field conditions. No field challenge of the enterotoxigenic *E coli* component was encountered, but experimental challenge studies with enterotoxigenic *E coli* have shown excellent protection (Snodgrass and others 1982a). In addition other K99-based vaccines have been reported to be effective in calves (Acres and others 1979, Nagy 1980). There is thus good reason to expect that the use of such a rotavirus/K99 vaccine can protect young calves against diarrhoea in a substantial proportion of herds where the problem is caused by rotavirus or enterotoxigenic *E coli* (Reynolds and others 1986, Snodgrass and others 1986). Subsequent to the trials reported in this paper this vaccine has been used as a whole-herd vaccine in approximately 50 herds with recurrent diarrhoea problems. No outbreaks of rotavirus or enterotoxigenic *E coli* diarrhoea have occurred in any of these herds, and cryptosporidiosis has been only an infrequent problem (unpublished information).

Acknowledgements.— The trial vaccine was generously supplied by Wellcome Biotechnology. The trial was possible only through the full collaboration and assistance given by many veterinary surgeons and their farmer clients, all of whom I gratefully thank.

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J. gen. Virol. (1984), **65**, 909–914. *Printed in Great Britain*

Vol. 20, No. 3

Key words: *rotaviruses, atypical/electrophoretypes/group*

Comparison of Atypical Rotaviruses from Calves, Piglets, Lambs and Man

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(*Accepted 8 February 1984*)

SUMMARY

Some rotaviruses from calves, piglets, and lambs were detected by electron microscopic examination of faeces but not by an enzyme-linked immunosorbent assay which relies on detection of group antigen. On further examination by polyacrylamide gel electrophoresis, these viruses had 11 segments of dsRNA, as had typical rotaviruses, but arranged in atypical patterns. From humans, three rotaviruses with atypical electrophoretypes were also detected. Gnotobiotic animals were infected with atypical calf, piglet and lamb rotaviruses, and used to provide antigen and antiserum for an immunofluorescent comparison of these rotaviruses with conventional rotaviruses and other previously described atypical rotaviruses from piglets and chickens. Two atypical rotaviruses from humans failed to infect gnotobiotic piglets. The atypical rotaviruses could be tentatively categorized into two groups serologically distinct from each other and from conventional rotaviruses, and these distinctions were consistent with electrophoretypes. The atypical chicken rotavirus may form a fourth distinct group. These findings are consistent with the hypothesis that rotaviruses belong to at least four separate groups definable by serology and electrophoretype.

INTRODUCTION

Most rotaviruses from mammalian and avian species share a common antigen demonstrable by techniques such as immunofluorescence, immune electron microscopy, complement fixation, and enzyme-linked immunosorbent assay (Woode *et al.*, 1976; Yolken *et al.*, 1978; McNulty *et al.*, 1979). Recently, however, viruses with characteristic rotavirus morphology which nevertheless lack this group antigen have been described from piglets (Bohl *et al.*, 1982; Bridger *et al.*, 1982), chickens (McNulty *et al.*, 1981), and children (Rodger *et al.*, 1982; Dimitrov *et al.*, 1983; Nicolas *et al.*, 1983). Although these antigenically distinct rotaviruses have 11 segments of dsRNA, their genome segment electrophoretic migration patterns are consistently different from those of conventional rotaviruses, and terminal fingerprint analysis also distinguishes the segments of the atypical rotaviruses (Pedley *et al.*, 1983).

As comparatively few of these atypical rotaviruses have been described, serological and genomic comparisons are limited (Pedley *et al.*, 1983). This paper describes such a study, using rotaviruses detected in the faeces of calves, piglets, lambs, and humans, and a chicken tissue culture isolate (McNulty *et al.*, 1981).

METHODS

Rotavirus strains. Faeces samples from diarrhoeic animals submitted to the Moredun Research Institute for rotavirus diagnosis were examined by electron microscopy (EM) (Snodgrass *et al.*, 1976) and enzyme-linked immunosorbent assay (ELISA) (Fahey *et al.*, 1981). Any samples that were rotavirus-positive by EM but negative by ELISA (and thus potentially missing the group antigen) were examined for their rotavirus dsRNA segment pattern by polyacrylamide gel electrophoresis (PAGE) (Herring *et al.*, 1982). Viruses showing atypical electrophoretypes were selected for further study. Stool samples containing atypical rotavirus from diarrhoeic humans were also included.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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A plaque-purified strain of the U.K. calf rotavirus (Bridger & Woode, 1975) grown in MA104 cells was used as a representative of conventional rotaviruses for serological tests, and a lamb rotavirus (Snodgrass *et al.*, 1976) as a conventional rotavirus for PAGE.

Animals. Gnotobiotic piglets, lambs, and calves were used. They were infected orally usually at 1 day of age with 2 ml of either 10 to 20% faecal suspension or faecal filtrate (0·45 μm). The animals were observed clinically, and faeces were collected daily. Some animals were killed after infection for collection of intestinal contents, and for preparation of intestinal sections from small and large intestine for histological and immunofluorescent examination (Snodgrass *et al.*, 1979).

Antisera. Experimental animals were bled for convalescent antiserum from 21 to 25 days after inoculation. In addition, hyperimmune antisera to some viruses were prepared by inoculating convalescent animals with virus purified from their own faeces by the method described in Ojeh *et al.* (1984), although yields of virus were poor due to the tendency of the atypical rotavirus particles to aggregate. Purified virus was emulsified in oil adjuvant and inoculated intramuscularly to animals 3 to 4 weeks after primary infection. Blood was then collected 2 weeks after hyperimmunization.

Sera were collected from patients JM and HM 5 and 6 weeks respectively after onset of illness diagnosed as an atypical rotavirus infection.

Other antisera used were: convalescent gnotobiotic calf and hyperimmune rabbit to conventional calf rotavirus; gnotobiotic piglet convalescent to the piglet rotavirus-like agent (Bridger *et al.*, 1982) supplied by Dr J. C. Bridger (Compton); gnotobiotic piglet convalescent to piglet pararotavirus (Bohl *et al.*, 1982) supplied by Dr L. J. Saif (Ohio); and chicken antiserum to chicken rotavirus 132 (McNulty *et al.*, 1981) supplied by Dr M. S. McNulty (Stormont).

Immunofluorescence (IF) tests. Test antigens used for our atypical rotaviruses were cryostat sections of small intestine. In addition, chicken rotavirus 132 grown in MA104 cells on coverslips was supplied by Dr M. S. McNulty, and cryostat sections of piglet intestine infected with piglet rotavirus-like agent were supplied by Dr J. C. Bridger. Calf rotavirus grown in MA104 cells on microtitre plates was used as an antigen representative of conventional rotaviruses.

Fourfold dilutions of each antiserum were tested with each antigen, with appropriate anti-species IgG conjugated with fluorescein isothiocyanate (FITC), except for antiserum to chicken rotavirus 132 which was conjugated with FITC for a direct IF test. Positive and conjugate controls were included in each test.

PAGE. Double-stranded RNA was extracted from faeces by the methods of Herring *et al.* (1982), and further purified by one cycle of CF 11 phosphocellulose chromatography (Franklin, 1966) performed as described by Bevan *et al.* (1973). Porcine pararotavirus dsRNA was kindly provided by Dr E. H. Bohl. PAGE was then performed using a 7·5% discontinuous gel; this was stained with silver (Ojeh *et al.*, 1984).

RESULTS

Detection of atypical rotaviruses by PAGE

Of 598 samples of calf faeces examined for rotavirus by EM and ELISA over 2 years, 179 were positive by both techniques, and 14 came into the category EM + ELISA −. Only two of these 14 had atypical electrophoretypes when examined by PAGE (D522 and D531). In addition, faeces samples from one piglet (D238) and multiple faeces samples from an outbreak of diarrhoea in lambs (E1101) also had atypical electrophoretypes. Stool samples from three humans (HM, JM and LK) from different sources also had atypical electrophoretypes. No viruses other than rotaviruses were detected by EM examination.

The genome profiles of the atypical rotaviruses were compared with conventional rotavirus (group A of Pedley *et al.*, 1983) and with porcine pararotavirus (group C) by PAGE (Fig. 1). The atypical rotaviruses had two distinctive electrophoretypes as described by Pedley *et al.* (1983). Both calf viruses (D522 and D531) and lamb virus E1101 were of the pattern described as group B, typified by close migration of segments 5 and 6 and the migration of segment 9 near 10 and 11. Piglet rotavirus D238 and the three human rotavirus strains were of the pattern described as group C, typified by segment 7 migrating near segments 5 and 6. However, as with conventional rotaviruses, minor variations within this overall pattern were apparent for each rotavirus strain.

Passage in experimental animals

Rotavirus D238 from a diarrhoeic piglet was given orally in faecal suspension to two piglets, and faecal filtrate from one of these piglets was used to infect four further piglets. All piglets

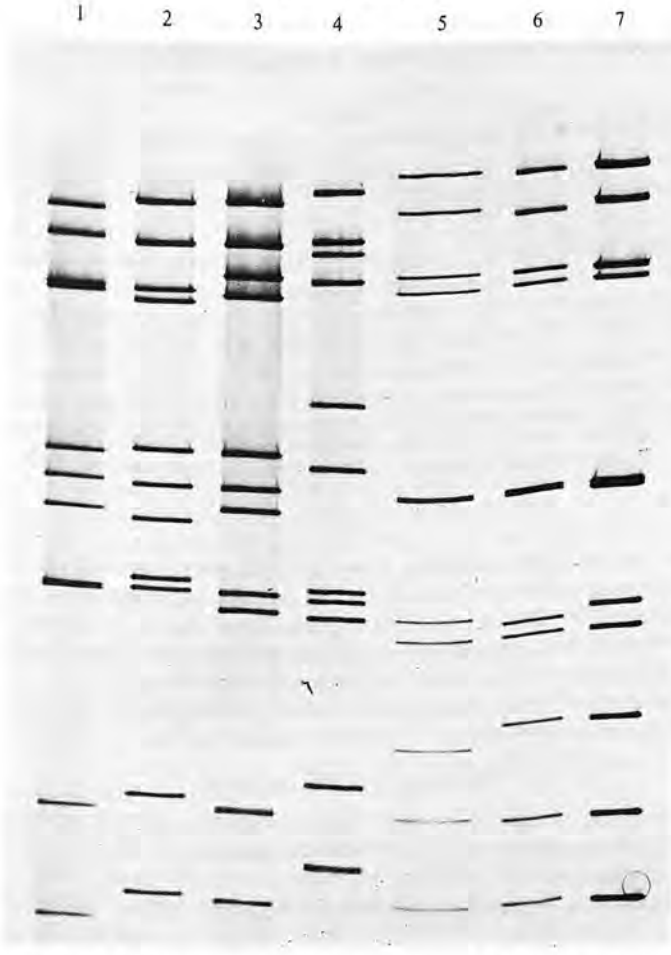

Fig. 1. Genome profile of rotaviruses by PAGE. Lane 1, human LK rotavirus; lane 2, piglet D238 rotavirus; lane 3, piglet pararotavirus (Ohio); lane 4, conventional lamb rotavirus; lane 5, calf D522 rotavirus; lane 6, calf D531 rotavirus; lane 7, lamb E1101 rotavirus. Lanes 1 to 3 contain rotaviruses exhibiting group C electrophoretype, lane 4 a typical group A electrophoretype, and lanes 5 to 7 group B electrophoretypes.

excreted D238 rotavirus in faeces within 24 h of infection. Severe diarrhoea, vomiting, anorexia, and dehydration occurred in piglets infected at 1 day of age, but milder diarrhoea only occurred in two piglets infected at 5 days old.

Rotavirus D522 from a diarrhoeic calf was given orally by faecal filtrate to one calf and two lambs, and faecal suspension from the calf was used to infect two further lambs. All animals excreted D522 rotavirus in faeces. The calf passed a large volume of loose yellow faeces on the day following infection, but was otherwise clinically normal. Only the lambs infected with the second passage level virus developed diarrhoea.

A suspension of lamb faeces containing rotavirus E1101 was given orally to one piglet. Intestinal contents from this piglet were given orally as suspension and filtrate to two and four further piglets respectively. Piglets excreted E1101 rotavirus in faeces within 17 h of inoculation, approximately coincident with the onset of a severe watery diarrhoea. Piglets infected at less than 3 days of age died within 48 h.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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Table 1. *Serological relationships (IF) between typical and atypical rotaviruses*

		Antiserum to rotavirus						
		Group A*		Group B		Group C		Group D
	Rotavirus	U.K.†	U.K.‡	D522§	Rotavirus-like (Compton)	D238‖	Pararotavirus (Ohio)	Chicken 132 (Stormont)
A*	Calf U.K.	2560	40	<10	<10	<10	10	<100
B	Calf D522	<10	<10	160	<10	<10	<10	<100
	Piglet rotavirus-like	NT¶	NT	160	160	NT	NT	NT
	Lamb E1101	<10	<10	640	<10	<10	NT	NT
C	Piglet D238	<10	<10	<10	<10	320	1280	<100
D	Chicken 132	<10	<10	<10	<10	<10	NT	>100

* Groups A, B, C, D: see text.
† Hyperimmune rabbit antiserum to calf rotavirus (U.K.).
‡ Convalescent gnotobiotic calf antiserum to calf rotavirus (U.K.).
§ Hyperimmune gnotobiotic calf antiserum to D522 rotavirus.
‖ Hyperimmune gnotobiotic piglet antiserum to D238 rotavirus.
¶ NT, Not tested.

Suspensions of human stools containing rotaviruses JM and LK were given orally to one and two piglets respectively. These piglets continued to pass formed faeces, and no rotavirus excretion was detected by EM or PAGE during the 7 days following infection.

Rotavirus D531 from a diarrhoeic calf was given orally as faecal filtrate to one lamb. The lamb excreted D531 rotavirus in faeces but remained clinically normal.

For each virus studied, the electrophoretic pattern detected by PAGE remained constant from the initial faecal sample through sequential passage.

Histopathological examination

Histopathological examination of intestine taken 2 days after infection from piglets infected with D238 and E1101 rotaviruses and from a lamb infected with D522 rotavirus revealed lesions of the small intestine typical of rotavirus infection (Snodgrass *et al.*, 1977). There were no abnormalities detected in the small intestine of a lamb killed 4 days after infection with D531 rotavirus.

IF examination

Immunofluorescence in small intestinal enterocytes with homologous antiserum was detected in piglets infected with D238 rotavirus, the most extensive staining being present in the duodenum of a piglet killed 15 h after infection. Immunofluorescence with homologous antiserum was detected in ileal enterocytes of a lamb killed 12 h after infection with D522 rotavirus. Immunofluorescence with antiserum to calf D522 rotavirus was detected in piglets infected with lamb E1101 rotavirus. Infected enterocytes were present throughout the small intestine in piglets killed at 11 h and 14 h, but not at 18 h after infection.

Serological comparisons

The serological comparisons using as antigen gut sections from the animals described in the previous paragraph are summarized in Table 1. Calf and rabbit antisera to conventional (group A) calf rotavirus reacted with U.K. calf rotavirus antigen, but with none of the atypical rotavirus antigens used.

Within the group B rotaviruses, antiserum to D522 calf rotavirus reacted with homologous antigen and with E1101 and piglet group B antigens. However, antiserum to piglet group B virus reacted with homologous antigen but not with either D522 or E1101 antigens. Neither of these antisera reacted with any other of the rotavirus antigens used.

Within the group C rotaviruses, antisera to D238 piglet rotavirus and to piglet group C rotavirus both reacted with D238 antigen, but not with any other of the rotavirus antigens tested.

Antiserum to chicken 132 rotavirus reacted only with homologous antigen.

Sera from humans HM and JM both had antibody titres to group A rotaviruses of 80 and to group B rotaviruses of <10. Titres to group C rotaviruses were 40 and 160 respectively.

DISCUSSION

The three criteria available for grouping atypical rotaviruses are serological distinction, distinct electrophoretype, and distinct RNA segment terminal fingerprint. Our results provide evidence that the atypical rotaviruses included in this study can be grouped on the basis of the first two of these criteria.

We detected two consistent atypical electrophoretypes, in both of which the typical group A rotavirus pattern of the 7th, 8th, and 9th segments migrating as a tight triplet was altered. Our D522 and D531 calf rotaviruses, and E1101 lamb rotavirus had electrophoretypes similar to the piglet rotavirus-like virus (Bridger *et al.*, 1982) proposed as group B by Pedley *et al.* (1983), with one of the triplet segments migrating more rapidly. Similarly our D238 piglet rotavirus and HM, JM, and LK human rotaviruses had electrophoretypes similar to piglet pararotavirus (Bohl *et al.*, 1982) proposed as group C (Pedley *et al.*, 1983), with one of the tight triplet segments migrating more slowly.

The serological results support this division of these atypical rotaviruses into two groups. Our D238 piglet rotavirus cross-reacted with piglet pararotavirus (group C), and our D522 calf rotavirus and E1101 lamb rotavirus showed a one-way cross-reaction with rotavirus-like virus (group B).

Thus there is good evidence provided by electrophoretype and serogroup that these atypical rotaviruses can be classified under the scheme of Pedley *et al.* (1983) as group B (D522 and E1101) and group C (D238). Others for which there is so far only electrophoretypic evidence can be more tentatively assigned to group B (D531) and group C (HM, JM, and LK). It will be interesting to compare the RNA segments by terminal fingerprint analysis (McCrae & McCorquodale, 1983) to ascertain if this method also can be consistently useful in atypical rotavirus classification, as so far only very limited data are available from one group B and one group C virus (Pedley *et al.*, 1983).

The presence of antibody in the human convalescent antisera to groups A and C but not to group B rotaviruses does not in itself indicate that these are group C rotaviruses, but is consistent with that hypothesis. So far all atypical human rotavirus strains appear from published electrophoretypes to fall within the group C rotaviruses (Rodger *et al.*, 1982; Dimitrov *et al.*, 1983; Nicolas *et al.*, 1983).

The failure of antiserum to piglet group B rotavirus to react with D522 and E1101 antigens is puzzling. It may reflect a greater specificity of the porcine serum for type-specific rather than group-specific antigens, with the existence of subgroups or serotypes within the group B rotaviruses as well as within the group A rotaviruses (Kapikian *et al.*, 1981).

Other variations within these groups of atypical rotaviruses are shown by the failure of human group C rotaviruses to multiply in gnotobiotic piglets. This is in contrast to the marked pathogenicity for piglets of group C rotaviruses isolated from pigs. Biological variations of this type may be as extensive among atypical viruses as in group A rotaviruses.

These studies confirmed that chicken 132 rotavirus does not cross-react serologically with rotaviruses of groups A, B, or C, and so is a potential group D rotavirus. The existence of at least four serologically distinct rotavirus groups poses an obvious diagnostic problem when techniques based on immunological specificity, such as ELISA, are used. This problem can be overcome with no loss of sensitivity by the use of diagnostic PAGE techniques (Herring *et al.*, 1982).

There are reasons to suppose that the atypical rotaviruses are comparatively uncommon: they remained undetected for many years in spite of widespread rotavirus research; they have been detected at levels varying from 0·25 to 4% of human rotavirus cases (Rodger *et al.*, 1982; Nicolas *et al.*, 1983; Dimitrov *et al.*, 1983); and we have detected only two atypical rotaviruses from 222 rotavirus-containing calf faeces ($<1\%$). On the other hand, antibody to groups B and C occurs commonly in cattle and pigs (Bridger *et al.*, 1983). This discrepancy could be accounted for if atypical rotaviruses were less virulent than group A rotaviruses, but evidence from this study and others suggests that group C rotaviruses are pathogenic for piglets (Bohl *et al.*, 1982; Bridger

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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et al., 1982), and one of our group B rotaviruses induced typical rotavirus lesions. Their overall importance remains to be established.

We gratefully thank those identified in the text for supplying us with viruses and antisera. Neil Inglis supplied able technical assistance, and K. W. Angus examined the histological sections.

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(*Received 6 December 1983*)

Archives of Virology 79, 161—171 (1984)

Paper 26

Archives of Virology

Paper 27

Vol. 20, No. 3

Evidence for Serotypic Variation Among Bovine Rotaviruses

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With 2 Figures

Accepted October 3, 1983

Summary

Eight field strains of calf rotavirus from the U.K. were compared by neutralisation tests, using convalescent and hyperimmune antisera. Seven of these strains cross-reacted and were considered to be of one serotype, while the 8th was distinguished by a greater than 20-fold two-way difference in neutralisation titre suggesting a second serotype. Three widely-distributed reference strains (U.K., Northern Ireland and Lincoln) cross-reacted with the strains in the dominant serotype, as did 33 of 42 other field calf rotavirus strains. Nine field strains failed to cross-react with either serotype, suggesting the existence of other potential serotypes in the calf population.

Introduction

Rotaviruses are a major cause of diarrhoea in young animals and children (9, 18). Calf rotavirus is of worldwide distribution, and in our experience is the single most important infectious cause of diarrhoea in calves (31).

The antigenic relationships between rotaviruses from all species are complex and there are at least 3 groups of rotaviruses which share no common antigens (5, 20, 25, 27). Recent results obtained with reassortant viruses (12) and monoclonal antibodies (11) have helped clarify the situation within the most common antigenic group. It has been suggested that the term subgroup be used to describe antigens detected by broad serological reactions involving the major structural protein of the core, using assays such as complement fixation, enzyme-linked immunosorbent assay, and immune adherence haemagglutination (15, 39, 40). To date, two subgroups of human rotavirus have been demonstrated (15). The term serotype defines antigens involved in neutralisation reactions, at least one of which is associated with the surface

reporting the existence of a third rotavirus serotype in the

respectively.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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glycoprotein of the outer shell (15). At least 4 and perhaps 5 human rotavirus serotypes exist (3, 36, 38).

Electrophoretic analysis of the eleven double-stranded RNA (ds RNA) viral genome segments has shown substantial variation in the pattern of segment mobilities (the electrophoretype) both between and within virus from different species (7, 14, 26, 29). Recent hybridisation and nuclease 'fingerprinting' studies have shown considerable sequence diversity even within ds RNA segments from strains of virus infecting one host species (27, 34). The non-cross-reactive groups of rotaviruses can be distinguished by electrophoretic analysis (5, 20, 25, 27), and the subgroups of human rotavirus can also be distinguished by their segment patterns (14).

Two serotypes of calf rotavirus distinguishable by neutralisation test have been reported from Japan (22), and some strains have been found distinct by haemagglutination inhibition test (33). Clearly serotype diversity is of great importance for potential vaccine development. The genome dsRNA pattern of bovine rotaviruses shows extensive variation (26). In this paper we describe investigations into the relationships by neutralisation assay and dsRNA electrophoresis of strains of rotavirus in calves in the United Kingdom.

Materials and Methods

Viruses

Faeces from twelve 1—4 week old calves which were shown by ELISA (8) and by electron microscopy (EM) (32) to contain rotavirus were initially selected for study. The basis for selection was the widely separate geographical origins within the U.K. of the faecal samples. Subsequently 42 other strains from calf faeces submitted to this laboratory for diagnostic examination were studied. Reference tissue culture adapted rotavirus strains used were U.K. (Compton) (6) (cloned by Dr. R. G. Wyatt), Northern Ireland (19) and the Lincoln Nebraska Calf Diarrhoea Virus (21).

Infection of Gnotobiotic Lambs

Gnotobiotic lambs reared singly in plastic isolators were used for the multiplication of the different isolates as well as for the production of specific convalescent antisera.

A 10 per cent suspension of each of the original faeces in 20 mM Tris HCl buffer pH 7.5 (Tris buffer) was blended with an equal volume of fluorocarbon (Arcton 113, ICI), centrifuged at $2000 \times g$ for 30 minutes, and the aqueous phase filtered through a 0.45 μm membrane. Three ml of filtrate was administered orally to each lamb 24 hours after delivery. Faeces were collected and examined daily for rotavirus excretion by ELISA and EM. Twenty-one days after inoculation the lambs were bled for antisera.

Tissue Culture

MA 104 cells used throughout the study were grown in Eagles 59 medium containing 10 per cent foetal bovine serum and maintained after inoculation in medium 199 containing 0.5 per cent BSA and 2 μg/ml trypsin (Sigma Chemicals Co. U.K.). All cultures were rolled after inoculation.

Tissue culture adapted strains of rotavirus were treated with 5 μg/ml of trypsin at 37° C for 30 minutes before inoculation. Virus stocks were produced by harvesting

infected cultures by 3 cycles of rapid freezing and thawing after 2—3 days or at maximum cytopathic effect (CPE).

To isolate rotavirus from field samples 0.5 ml of trypsin treated (10 μg/ml) faecal filtrate was inoculated to cell monolayers. Cultures were passaged at 3-day intervals and examined daily for CPE and at each passage by immunofluorescence for rotavirus. Isolates were cloned by passaging thrice at terminal dilution from the 6th or 7th passage level.

Rotavirus Purification

To prepare virus from the twelve gnotobiotic lamb faeces, samples were diluted in 3 volumes of Tris buffer, extracted with Arcton, and centrifuged at 2000×*g* for 30 minutes. Ten per cent sodium dodecyl sulphate was added to the supernate to a final 1 per cent v/v and incubated at room temperature for 5 minutes. This initial step was omitted when tissue culture grown rotavirus was used. Preparations were then pelleted at 71,000×*g* for 45 minutes. The pellets were homogenised in 1—2 ml Tris buffer, layered onto a discontinuous gradient consisting of 2 ml of a solution containing 1.31 M CsCl and 1.58 M sucrose, overlaid by 2 ml 1.58 M sucrose in Tris buffer and centrifuged at 154,400×*g* for 60 minutes at 5° C. The opalescent band which appeared just below the interface was harvested, diluted four-fold and pelleted. Pellets were resuspended in 1—2 ml of Tris buffer and layered onto a 5-step CsCl/sucrose gradient to which 1.0 μg/ml ethidium bromide had been added and then centrifuged at 50,400×*g* for 18 hours at 5° C. The gradient consisted of 1.66 M sucrose/1.49 M CsCl and 1.56 M sucrose/1.49 M CsCl at the extremities. An intermediate density was achieved by mixing equal volumes of the two extremes, and two further steps were achieved by mixing the intermediate solution with the two extremes. The virus band was located by fluorescence under ultraviolet light, harvested with a syringe, diluted in Tris buffer, and pelleted. The pellets were examined by EM using negative staining with 1 per cent ammonium molybdate (pH 6.0) and the proportion of complete virions estamined.

When tissue culture grown rotavirus was the starting material, virus was pelleted from the cleared supernate, and the resuspended pellet layered directly onto the 5-step CsCl gradient.

Hyperimmunisation of Rabbits

Purified virus pellets containing greater than 99 per cent complete virions were diluted to 1 ml in Tris buffer with 2 per cent Tween 80 added, and emulsified in incomplete Freund's adjuvant. Each rabbit (previously shown to be free of neutralising antibody to U.K. calf rotavirus) received a deep intramuscular injection of 1.0 ml of the emulsion at two different sites. The injections were repeated 14 days later and the rabbits were bled by cardiac puncture 7—10 days after the second injection.

Neutralisation Test

With both tissue culture adapted rotavirus strains and faecal rotavirus, neutralisation of fluorescent focus production in MA 104 cells was used, essentially as described by Beards *et al.* (3, 35). Titres (NT) are expressed as the reciprocal of the serum dilution reducing fluorescent foci by 50 per cent.

In the serotyping of field strains of rotavirus in faeces with standard rabbit serotyping antisera, a constant serum-varying virus assay was used. Faeces were extracted with fluorocarbon, mixed with antibiotics and centrifuged at 200×*g* for 30 minutes. Half $\log_{10}$ dilutions of the supernatant fluids were treated with trypsin at 10 μg/ml for 1 hour at 37° C, then incubated with 4 antibody units of typing antiserum for 1 hour at 37° C (1 antibody unit was the amount of antibody neutralised

reporting the existence of a third rotavirus serotype in ...

respectively.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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by 100 $TCID_{50}$ of homologous rotavirus), and thereafter assayed for fluorescent foci on MA 104 cells.

Polyacrylamide Gel Analysis (PAGE) of the dsRNA

Double stranded RNA was prepared from purified virions by phenol-chloroform extraction (13) and precipitation with alcohol and was fractionated in 7.5 per cent polyacrylamide gels with no stacking gel. The Laemmli buffer system (16) was used and electrophoresis was for 12 hours at a constant current of 0.1 mA/cm²; each gel track was loaded with 100—200 ng of dsRNA. After electrophoresis the gels were washed for 3—4 hours by gentle agitation in 5 changes of 1 per cent acetic acid/10 per cent ethanol and were then stained with silver as already described (13).

Results

Infection of Gnotobiotic Lambs

Rotavirus multiplication occurred in all 12 lambs, virus being excreted in faeces for at least 5 days. Coincident with virus excretion the faeces became loose and yellowish. A transient anorexia was noted in one lamb (678).

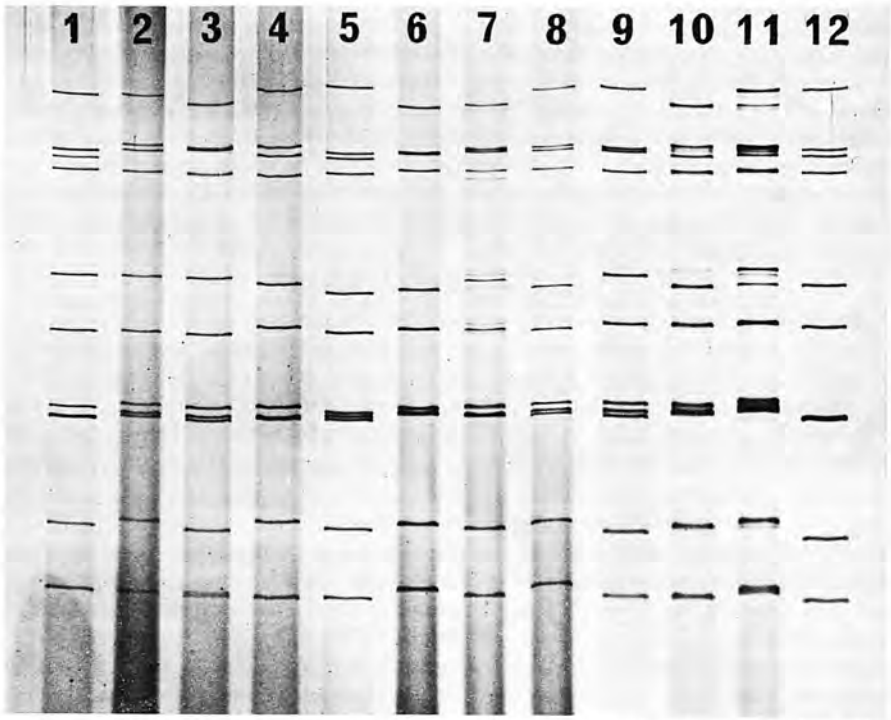

Fig. 1. Polyacrylamide-gel electrophoresis of dsRNA from 12 isolates. Tracks *1* 637; *2* 639; *3* 646; *4* 641; *5* 642; *6* 651; *7* 657; *8* 669; *9* 649; *10* 663; *11* 666; *12* 678. Note the existence of more than 11 segments in tracks 3, 7, 10 and 11

Examination of viral dsRNA by PAGE revealed that all 12 samples had different electrophoretic patterns. However, 4 of the samples (tracks 3, 7, 10 and 11) had more than 11 segments (Fig. 1). This was considered to reflect the presence of more than one strain of rotavirus in the original calf faeces, so subsequent studies were performed on the remaining 8 strains only.

Cell Culture Adaptation of Faecal Rotavirus

Two of the eight lamb-passaged calf rotavirus strains were selected for adaption to cell culture, 639 as typical serologically of 7 of the strains, and 678 as a distinct strain (see results below).

By the 4th passage level both strains produced slight CPE after 2 days incubation, and by the 6th passage this had developed to a CPE involving complete destruction of the monolayers after 3 days incubation.

The two isolates were cloned by three passages at terminal dilution. Examination of virus genome by PAGE at stages from faeces to cloned virus showed a consistent migration pattern within each isolate (Fig. 2) making the possibility of strain cross contamination unlikely.

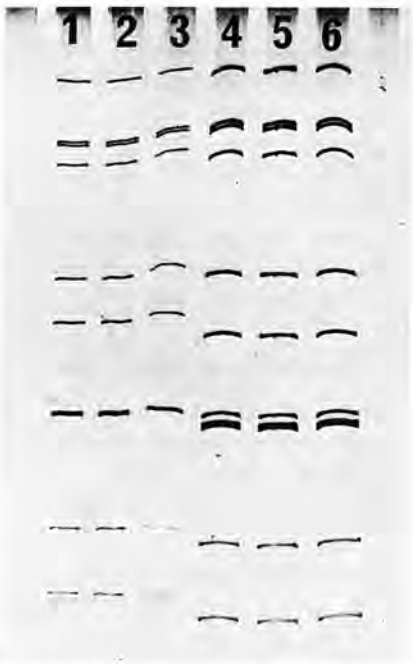

Fig. 2. Comparison of the dsRNA of 639 and 678 after adapation to tissue culture with dsRNA from the original faeces. Tracks *1* and *4* dsRNA of faecal 678 and 639 respectively; tracks *3* and *6* dsRNA of tissue culture adapted 678 and 639 respectively; tracks *2* and *5* a mixture of the dsRNA from faecal and tissue culture adapted virus

reporting the existence of a third rotavirus serotype in

respectively.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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Neutralisation Tests

Convalescent Lamb Antisera Reacted with Faecal Virus

All the lamb antisera possessed NT antibody to the homologous rotavirus strains, and also showed varying cross-neutralisation with other strains (Table 1). Within 6 of the strains 637, 639, 641, 649, 651 and 669 there was a high degree of cross-reactivity, with not more than an 8-fold variation between homologous and heterologous titres.

Table 1. *Neutralising titres of antisera from convalescent gnotobiotic lambs, to 8 strains of calf rotavirus in faeces*

	Antiserum to rotavirus							
Rotavirus	637	639	641	642	649	651	669	678
637	*128*	128	32	128	128	128	64	16
639	64	*128*	512	128	64	32	64	8
641	64	64	*64*	64	64	64	64	32
642	128	128	512	*1024*	128	128	128	32
649	128	128	32	ND	*128*	128	64	16
651	128	64	256	256	128	*64*	64	16
669	128	64	64	64	64	64	*128*	16
678	32	16	32	16	32	32	16	*256*

Homologous titres are in italics

ND not done

Antiserum to strain 678 showed 4 to 32 fold lower titres against all heterologous antigens, and 678 antigen was poorly neutralised by all other antisera. Strain 642 showed one-way cross-reaction with the group of 6 strains; 642 antigen was efficiently neutralised by other antisera, while 642 antiserum neutralised heterologous strains less efficiently.

Hyperimmune Antisera to Faecal Virus Reacted with Faecal Virus

The 6 strains considered similar in their reactions with convalescent lamb antisera were also similar in their reactions with hyperimmune antisera, with not more than a 4-fold variation in titre between homologous and heterologous strains (Table 2). Strain 642 appeared to be more closely related to the 6 strains in these tests, with efficient neutralisation of 642 virus by other antisera, and up to a 16-fold variation in titre of 642 antiserum with heterologous virus strains. Once again strain 678 showed significant distinction from all 7 other strains, with greater than 20-fold differences of titre in both directions.

This evidence suggested that seven strains including 642 were of one serotype, with less than 20-fold difference in titre, and that strain 678 was a distinct serotype.

Table 2. *Neutralising titres of hyperimmune rabbit antisera to faecal rotavirus strains, to 8 strains of calf rotavirus in faeces*

Rota-virus	Antiserum to rotavirus							
	637	639	641	642	649	651	669	678
637	*204,800*	102,400	51,200	102,400	409,600	51,200	25,600	6,400
639	204,800	*102,400*	102,400	204,800	819,200	102,400	25,600	3,200
641	102,400	51,200	*51,200*	51,200	204,800	102,400	25,600	6,400
642	409,600	102,400	204,800	*819,200*	12,800	204,800	51,200	6,400
649	204,800	51,200	204,800	819,200	*204,800*	51,200	51,200	1,600
651	51,200	51,200	204,800	409,600	819,200	*102,400*	51,200	3,200
669	102,400	51,200	51,200	204,800	204,800	ND	*51,200*	3,200
678	3,200	6,400	6,400	3,200	6,400	3,200	3,200	*102,400*

Homologous titres are in italics

ND Not done

Hyperimmune Antisera to Tissue Culture Virus Reacted with Tissue Culture Virus

The cell culture adapted U.K., Northern Ireland, and Lincoln strains were compared with 639 and 678 viruses isolated in cell cultures. The U.K., Northern Ireland and 639 strains appeared identical by cross neutralisation tests (Table 3). The Lincoln strain was less efficiently neutralised by antisera to these 3 strains, and thus showed slight one-way variation. However, the differences were never greater than 16-fold, so the Lincoln strain should still be considered as the same serotype.

Table 3. *Neutralising titres of hyperimmune rabbit antisera to rotavirus strains grown in tissue culture, to 5 strains of calf rotavirus in tissue culture*

Rotavirus	Antiserum to rotavirus				
	U.K.	Northern Ireland	Lincoln	639	678
U.K.	*5,120*	102,400	409,600	102,400	3,200
Northern Ireland	5,120	*102,400*	409,600	51,200	3,200
Lincoln	320	51,200	*409,600*	6,400	400
639	5,120	102,400	409,600	*102,400*	3,200
678	160	3,200	51,200	25,600	*204,800*

Homologous titres are in italics

Antiserum to 678 virus had heterologous titres in the range 64-512-fold less than the homologous titre although 678 virus was clearly distinguished by a greater than 20-fold titre difference by only 2 of the other antisera.

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respectively.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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Serotyping Faecal Rotavirus Strains

Due to the consistent distinction of 678 virus, it was considered to represent a separate serotype. Thus typing of field rotavirus strains was carried out using hyperimmune antisera to cloned 639 and 678 viruses. Allocation to serotype was on the basis of at least a 20-fold reduction in virus titre after incubation with the appropriate antiserum, compared with both the virus control titre and the titre with the other antiserum.

Sixty additional calf faeces samples containing rotavirus were examined. No typing of 18 strains was possible due to their failure to produce fluorescing foci in MA 104 cells. Antiserum to virus 639 neutralised the virus in 33 samples, while virus in 9 samples was not neutralised by either antiserum.

dsRNA Segment Pattern

The PAGE analysis of the viral dsRNA (Fig. 1) showed that all strains examined varied in their migration patterns, with no clear relationship to serotype.

Discussion

This study demonstrates the existence of two serotypes of rotavirus in calves. Whether the field viruses not neutralised by either antiserum were mixtures of more than one strain or represent potential new serotypes is currently under investigation. These two serotypes were defined by a neutralisation assay using fluorescent focus reduction with a 20-fold or greater two-way difference in titre as criterion for distinction (10, 38). The fact that the majority of our field calf rotavirus strains as well as three widely-used reference strains, all shared the same serotype, indicates that this serotype is at present the most common in the United Kingdom. This serotype, which we designated serotype 1, is probably similar to the serotype 1 proposed by Murakami *et al.* (22), as both were found similar to the Lincoln strain. The reference strain for serotype 1 could be either Lincoln, or the cloned U. K. strain used in our studies.

The *in vivo* active and passive immune relationships between the two serotypes are as yet unknown. Passive immunisation by dam vaccination is currently the most favoured method of prophylaxis in cattle (23, 30, 37). However, as passive immunity has been shown to be heterogenous between species by the protection of piglets against pig rotavirus infections using bovine colostrum (4, 17), the same broad passive protection may occur among serotypes of bovine rotavirus. In any case, the predominant occurrence of a single serotype suggests that in most instances a monovalent vaccine may be effective.

The techniques for cell culture isolation of rotaviruses from faeces used for calf rotavirus (1, 2) and for human rotavirus (28) proved successful in

this study. In addition to the two strains 639 and 678, four other strains that were not neutralised by antisera to either of the two serotypes have now been isolated and cloned. However, there was a degree of selection of suitable strains for culture, as 18 faecal samples containing rotavirus detected by EM and ELISA did not produce any fluorescent foci in MA 104 cells. The serotyping of such viruses presents a difficult problem.

Examination of the rotavirus genome dsRNA segments by PAGE provided a means of quality control by isolate identification throughout the isolation and cloning of field strains. Identical dsRNA migration patterns in original faeces and cloned virus make laboratory contamination very unlikely due to the great diversity of patterns seen in rotavirus surveys (24, 26). PAGE examination also detected mixed strain rotavirus infections in 4 of our original 12 faeces samples.

Acknowledgements

We thank Drs. Bridger, McNulty, and Wyatt for providing the reference viruses and Mr. McVittie and his assistants for feeding the lambs. We also acknowledge Messrs. B. J. Easter and A. Inglis for photographing the gels. C. K. Ojeh is in receipt of The British Council TCTD Award.

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Received July 25, 1983

Printed in Austria

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JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1984, p. 342–346
0095-1137/84/090342-05$02.00/0

Vol. 20, No. 3

Bovine Rotavirus Serotypes and Their Significance for Immunization

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Received 12 April 1984/Accepted 5 June 1984

Neutralization assays on calf fecal rotavirus with antisera to two previously described bovine rotavirus serotypes allowed the isolation of four rotaviruses belonging to a distinct third serotype. In a survey of 85 calf isolates, 80 rotaviruses belonged to serotype 1 (91%), 1 belonged to serotype 2 (1%), and 4 belonged to serotype 3 (5%). Serotypes 1 and 2 were shown to not cross-protect in a passive immunization experiment in gnotobiotic lambs. Ingestion of specific antiserum protected against infection with the homologous, but not heterologous, serotype. Rabbits with no previous exposure to rotavirus responded to sequential vaccination with bovine and human rotavirus serotypes with antibody specific to those serotypes, and the response did not broaden to include serotypes to which they had not been exposed. These factors suggested the need for multivalent rotavirus vaccines. By contrast, 47 adult cows on 11 farms had neutralizing antibodies to two bovine and three human rotavirus serotypes. After vaccination with one bovine rotavirus serotype, these cows produced a significant increase in antibody titers to these same five serotypes but not to two other serotypes to which they had no preexisting antibody. These results were interpreted to indicate that cows will respond heterotypically after monovalent vaccination to all rotavirus serotypes with which they have had experience and, therefore, that single serotype vaccination may be sufficient. This conclusion has practical importance for rotavirus immunization procedures.

The development of techniques for the routine isolation in cell culture of rotaviruses from feces of children and young animals (25) has facilitated the discrimination of serotypes as defined by neutralization assays (12). Several serotypes have been identified among rotaviruses from children (1, 30, 34), foals (8, 9), calves (11, 18, 21, 33), piglets (2), and avian species (17). The most thorough investigations have been made with human rotaviruses, and the existence of four distinct human rotavirus serotypes has been established (35). Simian, canine, and an equine (H-2) rotavirus have been found to be similar to serotype 3 of the human rotaviruses (9, 10, 34), whereas rotaviruses from foals (H-1) and calves (NCDV) were distinct from the human rotavirus serotypes (8, 34). Foal H-1 rotavirus was serotypically identical to three porcine rotavirus isolates (8).

No comparison has been made between the calf rotavirus serotypes from the United Kingdom, the United States, and Japan (11, 18, 21, 34). However, in each study the most commonly isolated rotavirus was serotypically related to the Lincoln strain of NCDV, so the characterization of bovine rotavirus serotype 1 would appear to agree.

The in vivo relationships of rotaviruses that are serologically distinct have been followed in sequential infection studies, in which it was shown that serotypically distinct isolates did not cross-protect (2, 4, 34). On the other hand, attempts are being made to use a calf rotavirus as a vaccine against human rotavirus infection (32, 37). However, as passive immunization via maternal colostrum and milk is now the most commonly practiced method of rotavirus vaccination in cattle (19, 23, 24, 27, 31), the practical significance of rotavirus serotypes in calves is best studied in passive rather than active immunization experiments.

This study continues our work on bovine rotaviruses by reporting the existence of a third rotavirus serotype in the cattle population of the United Kingdom and describes experiments assessing the significance of serotypes in passive immunization.

MATERIALS AND METHODS

Rotaviruses. The bovine rotavirus serotypes 1 and 2 (BRV1 and BRV2) previously characterized (21) were represented by UK and 639 isolates (BRV1) and 678 isolate (BRV2). Attempts were made to isolate in cell culture nine fecal calf rotaviruses that were not neutralized by BRV1 and BRV2 antisera (21). Four of these were successfully isolated and cloned by three passages at terminal dilution (411, 683, 2484, and 1548).

Human rotaviruses Wa, DS-1, M, and Hochi, representative of human virus serotypes 1 through 4 (HRV1 through HRV4), respectively (35), were kindly provided by R. Sanders of the Regional Virus Laboratory, East Birmingham Hospital, United Kingdom.

In the passive cross-protection experiment, the two challenge viruses used were: G753, the first gnotobiotic calf passage of a field calf rotavirus strain, which was shown to be identical to BRV1 by neutralization and to contain only a single rotavirus electropherotype, by polyacrylamide gel electrophoresis (PAGE) (6); and H799, the first gnotobiotic calf passage of calf rotavirus strain 678 (BRV2) which had been passaged once in a gnotobiotic lamb. Approximately 25% fecal suspensions were prepared in serum-free maintenance medium, homogenized with equal volumes of Arcton (Imperial Chemical Industries), centrifuged, and then sterilized by filtration through a 0.45-μm membrane. Lambs were infected orally with 5 ml of filtrate, and the titers of the inocula in MA104 cells were 4.6 and 5.3 $\log_{10}$ 50% tissue culture infective doses per ml for G753 and H799 viruses, respectively.

Antisera. Rabbits were hyperimmunized with tissue culture-grown rotaviruses as described previously (21). For the passive immunization experiment, sera from six rabbits

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immunized with 639 virus (BRV1) were pooled and sterilized by filtration, and a similar pool was made with antiserum to 678 virus (BRV2).

Serum samples were taken from 47 adult cows on 11 farms at the time of vaccination with an inactivated rotavirus-K99 *Escherichia coli* vaccine (27), and further serum samples were taken from the same cows 1 to 7 months later.

Cell culture and serology. All rotaviruses were propagated on MA104 cells as described previously (21). Neutralization tests were constant virus-varying serum tests on MA104 cells grown on microtiter plates. To characterize the rotavirus serotypes, fluorescent focus reduction tests were used (1). Subsequently, all tests with both bovine and human rotavirus isolates were read by cytopathic effect. Comparative titers were similar, but testing sera by fluorescent focus reduction tests gave titers approximately 100- to 1,000-fold higher. The antigenic relationship (R) between two strains of rotavirus was calculated with the formula $R = 100\sqrt{r1 \cdot r2}\%$ (29), in which r1 and r2 were the ratios of heterologous to homologous reciprocal antiserum titers for each antiserum tested with both viruses.

Additional bovine field strains were serotyped by neutralization assays of serial fecal dilutions after incubation with four antibody units of BRV1 or BRV2 antisera (21).

Passive immunization experiments. Twelve gnotobiotic lambs were allocated in pairs to six groups. Lambs were fed a normal diet of sterile evaporated milk diluted in water, were fed the appropriate serum dietary supplements, and were challenged with rotavirus (see Table 2). Serum was fed three times a day at 8-h intervals from day 2 to day 4 of life; 14 ml of the appropriate serum was incorporated in each feeding. Midway between feedings 1 and 2 on day 2 of life, or at the same age in the controls not fed serum, lambs were infected with the appropriate calf rotavirus.

Lambs were observed daily, with particular attention being paid to the consistency of the feces. Voluntary milk intake was recorded. Fecal samples were collected daily from each lamb and examined for the presence of rotavirus by enzyme-linked immunosorbent assay (ELISA) (3) and polyacrylamide gel electrophoresis (6, 21). Doubling dilutions of ELISA-positive samples were titrated by ELISA, and the titer was the highest dilution with an optical density at 405 nm greater than twice that of the negative feces samples. The serum-fed lambs were bled at days 5 and 23 of age, and the sera were titrated by immunofluorescent tests for antibody against UK calf rotavirus grown on MA104 cells in microtiter plates. Routine bacteriological examination of feces revealed that nine lambs remained bacteria-free throughout the experiment, and from three lambs non-enterotoxigenic *E. coli* organisms were isolated.

Sequential rabbit vaccination. Eight young rabbits with no antibody detectable by neutralization or immunofluorescent tests to any of our bovine or human rotavirus serotypes were vaccinated intramuscularly with 1 ml of rotavirus-*E. coli* vaccine containing BRV1 (27). Four of these rabbits (group A) were then revaccinated with BRV1 five times at monthly intervals. The other four rabbits (group B) were vaccinated sequentially at monthly intervals with BRV3, BRV2, HRV4, HRV3, and HRV2. Rabbits were bled initially, at the time of each vaccination, and 1 month after the final vaccination.

Control of cross-contamination by polyacrylamide gel electrophoresis. To avoid cross-contamination among the many rotavirus strains used, double-stranded RNA from all virus stocks was coelectrophoresed by polyacrylamide gel electrophoresis with the original bovine fecal viruses, or with the human viruses as initially received at the laboratory, as appropriate (6, 21). Fecal virus from the experimental lambs was similarly tested for identity with inoculum virus. Occasional cases of rotaviral contamination were detected in this way, and such stocks were discarded. The exception to this was HRV1 (Wa), which on receipt and throughout passage in our laboratory contained more than 11 segments of double-stranded RNA. We assumed that this was due to contamination with other rotavirus strains, but we were unable to obtain other stocks of HRV1. As this virus pool reacted serotypically like HRV1 and not with other serotype antisera, we used it, with some reservations, as HRV1.

RESULTS

Bovine rotavirus serotypes. The neutralization test results were expressed as R values to quantify more accurately the antigenic relationships of rotavirus isolates and to simplify presentation of the data. A 20-fold reciprocal difference in titer, suggested as the basis for rotavirus serotypic distinction (4, 34), is equivalent to $R = 5\%$, with greater R values signifying a closer degree of relationship.

The distinction of BRV1 (represented by UK and 639 viruses) and BRV2 (678 virus) was confirmed in this study, with $R < 2\%$ (Table 1). Three of the viruses that had not been typed by serotype 1 or 2 antisera (411, 683, and 2484) formed a closely related group ($R > 25\%$), which was clearly distinct from serotypes 1 and 2 ($R < 4\%$ in all except one result). These three viruses have been assigned to our serotype 3 (BRV3), to which virus 1548 may also belong ($R = 9$ to 18% with other BRV3 viruses).

A total of 108 calf fecal rotavirus samples have now been examined: 23 were untypable due to production of no or insufficient fluorescent foci on MA104 cells, and of the remaining 85, 80 belonged to BRV1 (91%), 1 belonged to BRV2 (1%), and 4 belonged to BRV3 (5%).

Passive cross-protection between serotypes. Control lambs infected with either BRV1 or BRV2 excreted rotavirus in feces from day 1 to days 5 through 7 after infection (Table 2). Lambs fed heterologous antiserum and infected with either serotype excreted rotavirus for a similar duration as the controls from day 1 to days 4 through 6 after infection. No rotavirus excretion was detected in lambs infected with BRV1 and fed type 1 antiserum, and the lambs infected with BRV2 and fed homologous antiserum excreted rotavirus only from days 6 through 10 after infection. Antibodies to rotavirus were detected by immunofluorescent test in the sera of all lambs by day 18 after infection.

Serological response of cows to vaccination. The 47 cows were selected at random from farms participating in a rotavirus vaccine trial. The mean prevaccination serum

TABLE 1. Cross-neutralization tests between serotypes 1 and 2 and the potentially different calf rotavirus serotypes

Rotavirus	Antiserum to rotavirus						
	Serotype 1		Serotype 2	Potential new serotypes			
	UK	639	678	411	683	2484	1548
UK	100[a]						
639	25	100					
678	<1	2	100				
411	2	3	2	100			
683	3	2	3	100	100		
2484	4	2	9	25	25	100	
1548	3	3	2	9	13	18	100

[a] R% (defined in the text).

TABLE 2. Passive cross-protection experiment[a]

Infection serotype	Antiserum serotype	Lamb no.	Virus excretion (ELISA titer) at days post inoculation:									
			1	2	3	4	5	6	7	8	9	10
1	None	00	32	32	32	128	32					
		01	64	64	32	16	16	8				
2	None	02	2,048	2,048	1,024	256	64	32	16			
		03	2,048	2,048	2,048	64	32					
1	2	19	512	512	512	512	32					
		20	512	512	512	512	512	32				
2	2	17							512	512	512	32
		18							32	512	512	32
2	1	15	32	64	128	32						
		16	32	32	256	512	512	32				

[a] Rotavirus excretion monitored by polyacrylamide gel electrophoresis demonstrated identical duration of virus excretion. No virus was detected at any day post inoculation for lambs 13 and 14 with infection serotype 1 and antiserum serotype 1.

neutralizing antibody titers of the cows to BRV1 and BRV2 and to HRV1, HRV2, and HRV3 ranged from 42 to 80 (Table 3). Although the interval between vaccination and serum sampling varied from 1 to 7 months on the different farms, there was no significant between-farm variation in these prevaccination titers. The prevaccination titers in all the animals from all the farms to BRV3 and HRV4 were low or negative, with only 5 and 15 of the animals, respectively, having detectable antibody (>10).

After vaccination with BRV1, the responses of the cows to the different serotypes of bovine and human rotaviruses were proportional to the initial prevaccination titers. There were significant seroconversions of similar magnitude to each of BRV1 and BRV2 and HRV1 and HRV3, and a slightly lower response to HRV2 ($P < 0.05$). There was no response to BRV3 or HRV4 even in the minority of cows which had low prevaccination titers.

Sequential vaccination of rabbits. The results with cattle vaccination led us to investigate a rabbit model. No antibody to any rotavirus serotype was detected before vaccination in sera from any of the eight rabbits. After initial vaccination with BRV1, all eight rabbits produced neutralizing antibody specific to that serotype but to no other serotype. The four rabbits which were vaccinated with BRV1 on five further occasions maintained BRV1 antibodies throughout the experiment, but did not produce antibodies to any of the other serotypes (Table 4). The four group B rabbits which were revaccinated at monthly intervals with different serotypes produced antibodies to the most recently administered serotype on each occasion, but did not on any occasion produce antibodies to rotavirus serotypes to which they had not been previously exposed.

DISCUSSION

The existence of a distinct third serotype of rotavirus isolated from calves in the United Kingdom was clearly demonstrated in this study. Rotaviruses 411, 683, 2484, and 1548 were distinct from the previously described BRV1 and BRV2 rotavirus serotypes (21) with R values of 2 to 4% against BRV1 and 2 to 9% against BRV2. There was a high degree of relatedness among three of these serotype 3 viruses ($R > 25\%$), but the fourth virus in this serotype (1548) was less closely related to the others ($R = 9$ to 18%).

In adult cows there was a uniformly widespread occurrence of antibody to BRV1 and BRV2 and to HRV1, HRV2, and HRV3, but not to BRV3 or HRV4. The cows responded to monovalent vaccination with BRV1 with a significant increase in antibody only to the five serotypes to which they had preexisting antibody. Interpretation of these findings is aided by the results of the experiment in which rabbits were vaccinated sequentially with different bovine and human rotavirus serotypes. These rabbits responded with antibody to vaccine virus serotype, but not to any serotype of which they had no previous experience. Similarly, rabbits vaccinated with BRV1 on six separate occasions produced antibody specific only to that serotype. These results suggest that repeated exposure of animals to different rotavirus serotypes results in acquisition of antibody specific to each serotype, but that the response does not broaden to include serotypes of which the animal had no previous experience. Similarly, the response of gnotobiotic calves to human rotavirus was largely serotype specific (36), and a type-specific response was observed in mice, although it was not certain whether they had previous exposure to other rotaviruses (20). In humans with presumed previous multiple exposure to rotavirus serotypes, infection resulted in both homotypic and heterotypic increases in antibody titer (5, 13).

Heterotypic immunity has been well defined in influenza A infections (14). Antibody response to specific hemagglutinins of unrelated subtype was enhanced by previous infection with heterotypic viruses with common internal antigens, possibly by T helper cells which recognized common antigens cooperating with B cells which recognized specific

TABLE 3. Mean serum neutralizing antibody titers to seven rotavirus serotypes of 47 cows before and after vaccination with BRV1

Neutralization test antigen	Prevaccination titer	Postvaccination titer
BRV1	68	640
BRV2	64	650
BRV3	7	5
HRV1	80	498
HRV2	42	281
HRV3	53	528
HRV4	10	11

TABLE 4. Mean neutralizing antibody response of rabbits to different rotavirus serotypes[a]

Rabbit group	Antigen	Mean neutralizing antibody titer ($\log_{10}$) of sample:					
		2	3	4	5	6	7
A	BRV1	2.43	2.51	2.51	2.88	2.73	2.58
	All others	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
B	BRV1	2.35	3.03	2.88	2.96	2.51	2.42
	BRV3	<1.0	2.81	3.11	2.88	2.66	2.35
	BRV2	<1.0	<1.0	3.18	2.73	2.66	2.66
	HRV4	<1.0	<1.0	<1.0	2.13	2.05	2.05
	HRV3	<1.0	<1.0	<1.0	<1.0	2.20	2.20
	HRV2	<1.0	<1.0	<1.0	<1.0	<1.0	2.05
	HRV1	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

[a] Samples 1 through 6 were collected from group A rabbits at times of sequential vaccination with BRV1, and sample 7 was collected 1 month after the last vaccination. Samples 1 through 6 were collected from group B rabbits at the time of vaccination with BRV1, BRV3, BRV2, HRV4, HRV3, and HRV2, respectively, and sample 7 was collected 1 month after HRV2 vaccination. Mean neutralizing antibody titer of sample 1 was always <1.0.

hemagglutinin (22). If this suggestion that heterotypic immunity depends on complete virus as antigen can be confirmed, conventional tissue culture-derived vaccines may have a considerable advantage over potential vaccines based on single proteins. However, other mechanisms may be involved in rotavirus immunity, as heterotypic neutralizing activity has been described after immunization of guinea pigs with polypeptides alone (15).

The serological results suggest that there are at least five serotypes of rotavirus endemic in the cattle population which are antigenically similar to BRV1 and BRV2 and to HRV1, HRV2, and HRV3. These results are partially at variance with the findings from serotype identification of rotaviruses from calf feces, in which BRV1 was of common occurrence, BRV3 was much less common but nevertheless four isolates from separate farms were made, BRV2 was isolated from only one calf, and no viruses similar to HRV1 through HRV3 were identified. There are two possible explanations for these differences. First, some serotypes may not readily infect MA104 cells and thus would escape detection by the techniques used in this study. There is some evidence to support such variation, as rotaviruses from calves have consistently been more readily cultivated in vitro (16). Alternatively, there may be differences in epidemiology and pathogenesis of different serotypes, such that some are commonly associated with diarrhea in young animals, whereas others may infect older age groups or be responsible for subclinical infections. This would be analogous to the situation with rotaviruses infecting children, in which those infecting neonates may often belong to HRV3 or HRV4 (35). Thus these observations of different rotavirus serotype prevalences in serum surveys and diarrheic feces may not be contradictory.

Experimental vaccination of pregnant cows with rotavirus vaccines in adjuvant has consistently caused antibody excretion in colostrum and milk for several weeks after calving (7, 24, 26). However adequate data on the ability of these vaccines to prevent diarrhea caused by rotavirus in the field are relatively scarce. In a field trial of a rotavirus-K99 vaccine (27), we have demonstrated statistical evidence of efficacy in dairy herds in which 50% of the cows were vaccinated, and we have not encountered any rotavirus diarrhea problems in 30 beef herds in which all cows were vaccinated (unpublished data). This is in contrast to the situation in unvaccinated herds in which over 50% of all diarrhea outbreaks in calves were caused by rotavirus (28). Similar suggestive evidence of rotavirus vaccine efficacy has been obtained in France (19) and Belgium (31). Thus the demonstration that antiserum to one serotype of rotavirus does not protect against infection with a different serotype is of considerable importance, particularly as previous observations that rotavirus serotypes do not cross-protect have been derived from active immunity experiments (2, 4, 34). At first sight, these results imply that calf rotavirus vaccines would be required to contain all serotypes commonly found in diarrheic calves. That this may not be so can be deduced from examination of the serological response of cows to monovalent vaccination, in which it was demonstrated that the cows responded heterotypically to all serotypes of rotavirus to which they had previously been exposed. In these circumstances, vaccination with one serotype may be satisfactory, a suggestion supported by the generally beneficial results achieved with a vaccine containing only BRV1 (19, 27, 31).

ACKNOWLEDGMENTS

C.K.O. received a British Council TCTD award.
We thank M. McLachlan for help with the statistical analysis.

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Archives of Virology 55, 287—291 (1977)

Archives of Virology

Detection and Transmission of 30 nm Virus Particles (Astroviruses) in Faeces of Lambs With Diarrhoea

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With 2 Figures

Accepted July 5, 1977

Summary

An outbreak of diarrhoea in lambs was investigated, and electron microscopic examination revealed small round virus-like particles in the faeces from eight of seventeen lambs. A bacteria-free filtrate of faeces from one lamb was given orally to a gnotobiotic lamb, which subsequently excreted virus in faeces. Intestinal contents were collected from this lamb and a filtrate given orally to two further gnotobiotic lambs, which subsequently developed diarrhoea and excreted virus in faeces. The mean diameter of the virus particles was 29.7 nm, and 12 per cent of them showed their surface structure arranged in the form of a six-pointed or five-pointed star. They were similar to those particles previously observed in human infant faeces which were referred to as "astroviruses".

The passage through gnotobiotic lambs with development of diarrhoea showed that these particles were animal viruses which were probably pathogenic for lambs.

Introduction

Direct electron microscopic (EM) examination of faeces from man and animals has resulted in the acceptance of rotaviruses as a cause of neonatal diarrhoea (1). In addition to rotaviruses, small round virus-like particles have been observed by EM examination of stools from cases of infectious non-bacterial gastroenteritis in man. In most instances, these have been 22—28 nm in diameter with no distinguishing morphological features. A serological relationship between some of these particles has been shown by immune EM techniques (2, 10), and by cross-challenge experiments in volunteers (12). Some have been suggested as parvovirus-like (3, 10), others as probable enteroviruses (3, 7).

There are other reports of the observation in human stools of small round virus-like particles with a more characteristic structure; some with a similarity to

caliciviruses (6), and others, 28 nm in diameter, with a five- or six-pointed stellate configuration superimposed on the circular shape, for which the name astrovirus was suggested (4, 5). There are at the moment no reports of astroviruses in faeces of species other than man. We record their detection in faeces from diarrhoeic lambs, and their successful transmission to gnotobiotic lambs with the production of diarrhoea.

Materials and Methods

Outbreak of Diarrhoea in Lambs

Diarrhoea occurred in a group of 4–6 week old Suffolk lambs on a commercial farm. *Escherichia coli* of unknown enteropathogenicity was isolated from the diarrhoeic faeces, and helminth egg counts and coccidial oocyst counts were below clinically significant levels. A viral involvement in the aetiology of this diarrhoea was considered, and investigations undertaken.

Electron Microscopy

Faeces from both naturally- and experimentally-infected lambs were mixed with distilled water to give an approximate 20 per cent suspension. The large debris was allowed to settle before a drop of the supernatant fluid was transferred to a carbon collodion-coated grid and stained with either 1 per cent potassium phosphotungstic acid (pH 7.0) or 1 per cent ammonium molybdate (pH 5.3).

Infection of Experimental Lambs

Virus-containing faeces from one naturally-infected lamb were diluted to 20 per cent in distilled water, and filtered through a 0.22 μm membrane. 1 ml of this filtrate was given orally to a 2-day-old gnotobiotic lamb (No. 1), which was killed on the third day after infection and the intestinal contents collected. A bacteria-free 20 per cent filtrate of these intestinal contents was prepared in a similar manner and given orally in 3 ml amounts to 3-day-old gnotobiotic lambs (Nos. 2 and 3). Lamb 2 was killed on the 4th day after infection. Lamb 3 was bled for antiserum preparation 18 days after infection.

No bacteria were isolated from lamb 1, and a *Bacillus* sp was isolated from faeces of lambs 2 and 3. No pathogenic significance is attached to this organism.

Cell Culture

Filtrates of intestinal contents were inoculated onto foetal lamb kidney (FLK) cells which were grown and maintained as described (9). Cells from five successive virus passages were examined for cytopathic effect, and were also examined by immunofluorescence using antiserum from lamb 3. In addition, filtrates of intestinal contents were centrifuged in microtitre plate wells containing FLK cells (8). After incubation for 24 hours at 37° C, these cells were examined in an immunofluorescence test using antiserum from lamb 3 or gnotobiotic lamb antiserum to lamb rotavirus (9).

Results

Outbreak of Diarrhoea in Lambs

By electron microscopic examination, low numbers of small round virus-like particles with the morphology of astroviruses (5) were seen in faeces from eight of seventeen lambs examined.

Clinical Response to Experimental Infection

Lamb 1 remained clinically normal, but passed faeces that became loose though not diarrhoeic. Faeces from lambs 2 and 3 became loose in consistency on

the third day after infection, and a yellowish diarrhoea developed in both lambs on the fourth day, persisting for two days in lamb 3. No other clinical abnormality was noted in either lamb.

Virus Excretion

Small, round virus-like particles were observed in the faeces of lamb 1 on the third day after infection. On the second lamb passage, similar particles were observed in the faeces of lamb 2 from the second day after infection. These were present in large numbers in the contents of the large intestine at necropsy, but only in small numbers in the contents of the small intestine. Lamb 3 excreted similar virus-like particles from the 3rd to 9th days after infection.

Virus Morphology

The virus-like particles observed in faeces and intestinal contents were roughly circular in outline (Fig. 1). About 12 per cent of the particles showed their surface structure arranged in the form of a six-pointed or occasionally five-pointed star. Bridging structures were frequently seen between adjacent particles (Fig. 2). This morphology is similar to that described by MADELEY and COSGROVE (5) for human astroviruses, although there was no evidence of quasicrystalline arrays in our preparations. The similarity of the lamb virus to the astroviruses observed in human stools has been confirmed (C. R. Madeley personal communication). The

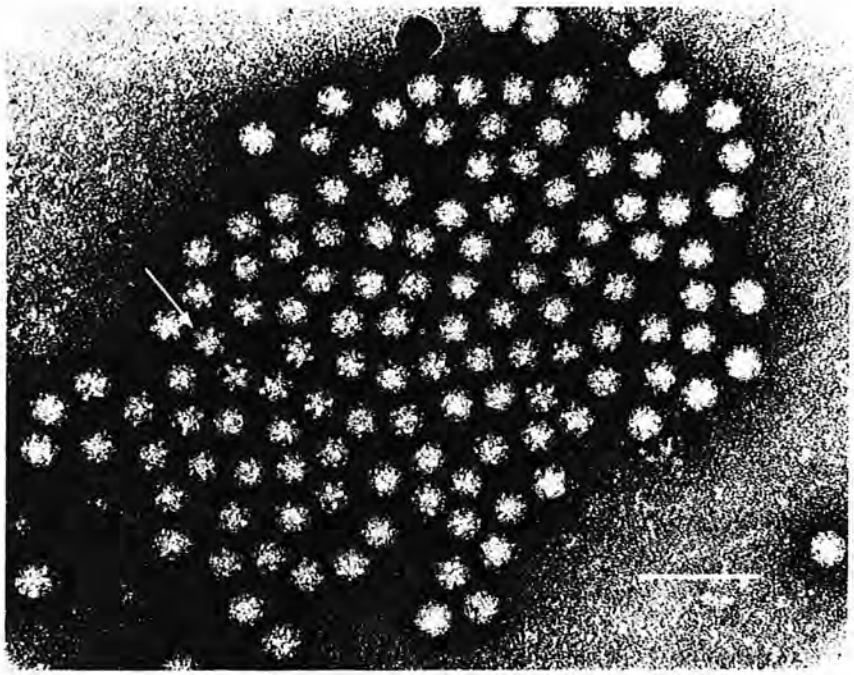

Fig. 1. Astrovirus particles in intestinal contents of lamb 1. The arrow indicates a group of particles showing star-like surface structure. Bar represents 100 nm. Stained ammonium molybdate

size of the virus particles was determined by comparing them with the lattice spacing of beef-catalase crystals photographed in the same field (11). The mean size of 25 particles was 29.7 ± 0.8 nm.

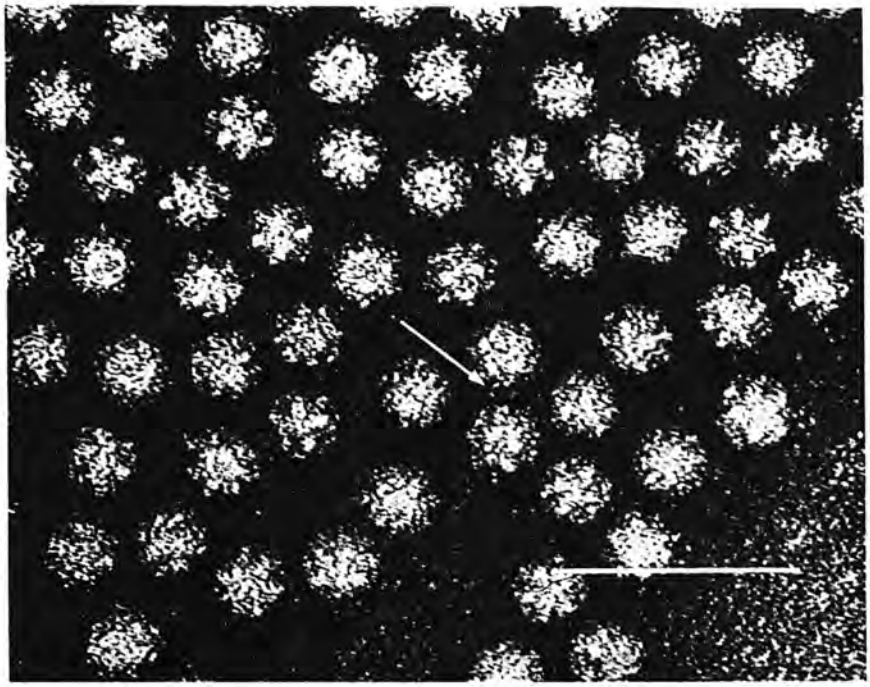

Fig. 2. Astrovirus particles in intestinal contents of lamb 1. The arrow indicates structures bridging particles. Bar represents 100 nm. Stained ammonium molybdate

Cell Culture

No evidence of virus multiplication in FLK cells was obtained by examination for cytopathic effect or specific immunofluorescence in tube cultures or microtitre plate wells. There was no immunofluorescence in cells inoculated with astrovirus filtrate and tested with rotavirus antiserum, or between FLK cells containing lamb rotavirus which were tested with antiserum from lamb 3.

Discussion

The virus studied in these experiments appears to conform to the type of small round viruses described as astroviruses (5). The only criteria at present for this classification are stellate morphology, a size of approximately 30 nm, and occurrence in faeces. All of these are fulfilled in the case of this lamb virus. Whereas Madeley and Cosgrove (4) could not be certain that these viruses were not bacteriophages, their transmission through gnotobiotic lambs in this case shows that they are animal viruses. Their structure appears unique, but their relationships and classification have to await further study.

The experimental lambs excreted astrovirus in faeces for several days, and in the case of lamb 2 had a large volume of intestinal contents containing astrovirus at necropsy. This indicates that the virus detected resulted from replication and was not merely residue from the small volume of inoculum. No other virus was observed in lengthy electron microscopic examination of faeces samples from the experimental lambs; no cytopathic virus was isolated in FLK cells; and the presence of rotavirus was specifically excluded by crossimmunofluorescence. Thus it is probable that the clinical disease observed in the experimental study was caused by the astrovirus, and that this virus can be considered an enteric pathogen of lambs. The natural occurrence of astrovirus and its role in aetiology of diarrhoea are unknown, although the temporal association of the astrovirus with diarrhoea in eight of the seventeen lambs examined in the original outbreak suggests an aetiological link in this case.

Acknowledgments

We thank Mr. R. Clutterbuck and Mr. S. Price of Much Wenlock, Shropshire, for bringing the original outbreak to our notice, and P. Gray and M. Robertson for excellent technical assistance.

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Received June 20, 1977

Printed in Austria

Archives of Virology 60, 217—226 (1979)

Archives of Virology

Pathogenesis of Diarrhoea Caused by Astrovirus Infections in Lambs

By

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With 6 Figures

Accepted January 23, 1979

Summary

Experimental infection of 2-day-old gnotobiotic lambs with lamb astrovirus produced mild diarrhoea after an incubation period of about 48 hours. No other clinical symptoms developed. Infection was studied by immunofluorescent and histological examination of tissues from the lambs. Astroviruses infected only mature villus epithelial cells and subepithelial macrophages in the small intestine, where they produced partial villus atrophy. Infected enterocytes were replaced with cuboidal cells from the crypts, and the lesion gradually healed by 5 days after infection. No serological relationship was detected by immunofluorescence between lamb astrovirus antigen in gut sections and antisera to either calf or human astrovirus.

Introduction

Viruses of 28—30 nm diameter with a circular outline and stellate surface structure have been observed in faeces from diarrhoeic children (7, 14), lambs (15) and calves (19). The name astrovirus has been suggested for these morphologically distinctive viruses (6) and will be used in this paper.

Little is known of the pathogenic potential of these viruses. After oral inoculation they produced mild diarrhoea in lambs (15), and partial villus atrophy in calves (19) and inconsistently caused diarrhoea in adult human volunteers (5). This paper reports findings made on the pathogenesis of astrovirus infections in gnotobiotic lambs.

Materials and Methods

Infection of Lambs

Six gnotobiotic lambs were infected orally when approximately 24 hours old with intestinal contents from the third passage of lamb astrovirus in gnotobiotic lambs (15). Five of the lambs received 1.5 ml of a 0.22 μm filtrate of a 20 per cent suspension of

0304-8608/79/0060/0217/$ 02.00

these intestinal contents, while the sixth (that killed 120 hours after infection) received 1.5 ml of ulfiltered bacterin-free contents. The inoculum appeared by electron microscopic examination to contain many fewer astroviruses after filtration. One lamb was killed at each of the following hours after infection (p.i.): 14, 23, 38, 45, 70 and 120. Five gnotobiotic lambs killed between 72 and 144 hours of age served as controls for the histology. Six gnotobiotic lambs between 72 and 408 hours of age were controls for lactase estimations.

Necropsy Procedures

Lambs were deeply anaesthetised with sodium pentobarbitone or halothane. Segments were obtained from jejunum (about 10 cm distal to the duodeno-jejunal flexure); from midgut; and from ileum (about 50 cm proximal to the ileo-caecal junction, from an area free of Peyer's patches). The lambs were then killed by exsanguination, and tissues collected from caecum, colon, kidney, liver and lung.

Histological Methods

Tissues for histological examination were fixed in 10 per cent formalsaline and processed as described previously (16). Additional small (1 mm^3) blocks of intestine were fixed in 3 per cent glutaraldehyde in phosphate buffer (pH 7.4) and processed to Araldite. Sections 1 μm thick were cut and stained by 10 per cent Giemsa, at 60° C.

Villus heights and crypt depths in HE-stained sections were measured by ocular micrometer on ten vertically-cut, full length villi and crypts at each site of small intestine.

Immunofluorescence

Additional portions of all tissues were frozen in a CO_2-ethanol freezing mixture. Lengths of small intestine and colon were filled with embedding medium (Tissue-Tek II, Lab-Tek Products) prior to freezing, to aid proper orientation of villi. Frozen tissues were mounted on microtome chucks and 6 μm sections cut on a cryostat. An antiserum to lamb astrovirus was prepared as follows: a gnotobiotic lamb was infected orally with astrovirus, and reinfected 10 days later. After a further 4 days the lamb was given by intramuscular inoculation an astrovirus preparation partially purified by differential centrifugation of intestinal contents. Blood was collected for serum preparation 5 days after final inoculation. Tissue sections were treated with this antiserum, followed by fluorescein-conjugated rabbit anti-sheep globulin. Control sections were treated with gnotobiotic lamb antiserum to lamb rotavirus, followed by the conjugated anti-sheep globulin.

For purposes of serological comparison, astrovirus-containing gut sections were stained with calf antiserum to calf astrovirus (kindly supplied by Dr. J. C. Bridger, Compton, Berkshire) or human antiserum to human astrovirus (kindly supplied by Dr. J. B. Kurtz, Churchill Hospital, Oxford), followed by the appropriate fluorescein-conjugated globulins.

Enzyme Analysis

Additional portions of tissue from the three sites of small intestine were collected and stored at —20° C. Lactase estimations used the methods of Dahlqvist (1).

Virus Detection

Faeces samples were collected daily from all lambs, and suspensions examined by electron microscopy for the presence of astrovirus (15).

Results

Clinical and Virological

The lambs killed at 14, 23 and 38 hours p.i. did not develop diarrhoea. The other three infected lambs developed diarrhoea 44—48 hours after infection, faeces changing from firm and dark brown in character to very loose and yellow.

Voluntary milk intake remained normal. Astrovirus was not observed in faeces from the lambs killed 14 and 23 hours p.i., but was first seen in the faeces of all other infected lambs between 38 and 48 hours p.i. At necropsy, astrovirus was detected in intestinal contents of all lambs except that killed 14 hours p.i.

The control lambs remained clinically normal, and passed firm brown faeces throughout the duration of the experiment. No virus particles were detected in their faeces.

Immunofluorescence

Specific immunofluorescent staining was detected only in scattered epithelial and subepithelial cells on small intestinal villi (Fig. 1). The immunofluorescence was usually fine and stippled in appearance (Fig. 1 b). Infected cells were present between 14 and 70 hours p.i. (Table 1). Fewer infected cells were present in jejunum than in midgut or posterior ileum. The greatest number of infected cells was present early in infection, during the incubation period from 14 to 38 hours p.i. The infected enterocytes were generally scattered through the apical half of the villi. Occasional infected cells were observed in the villus lamina propria. No specific reaction was observed when the rotavirus antiserum was used. Tissues other than small intestine showed no specific reaction.

No immunofluorescence was observed with lamb astrovirus-infected intestinal sections and the antisera to either calf or human astrovirus.

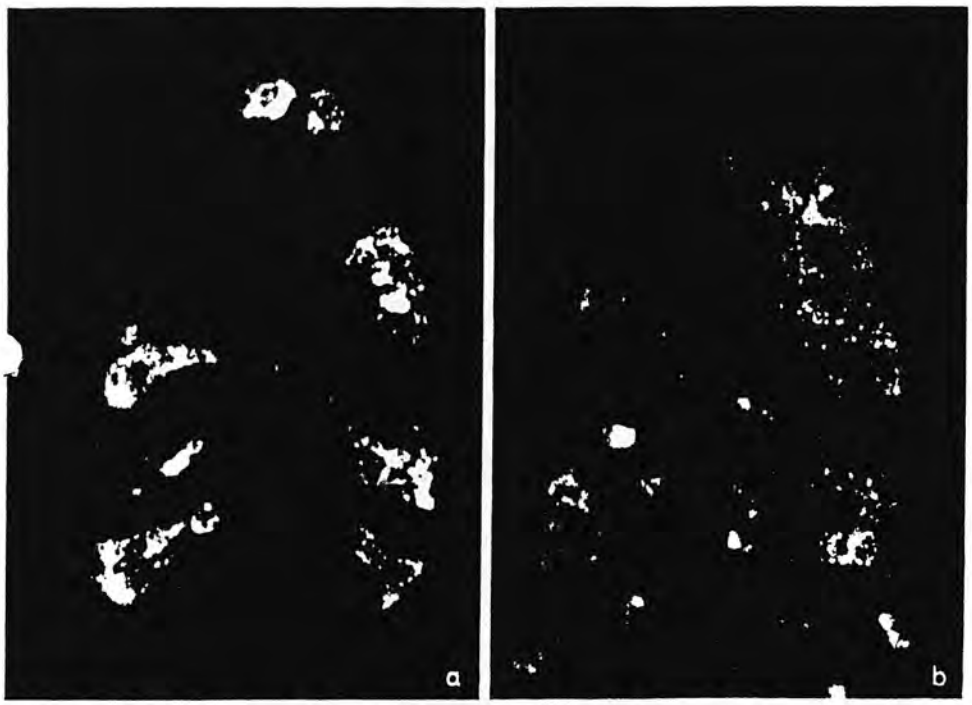

Fig. 1. Astrovirus immunofluorescence in midgut. *a* 23 hours p.i., transverse villus section. *b* 38 hours p.i., longitudinal villus section

Table 1. *Astrovirus immunofluorescence detected in the small intestine*

Time killed (hours p.i.)	Site		
	Jejunum	Midgut	Posterior ileum
14	++	++	++
23	+[a]	++[b]	+++[c]
38	+	+++	++
45	+	+	−
70	+	+	−
120	−	−	−

[a] + Very occasional infected epithelial cells (less than 1 per villus section)
[b] ++ Infected cells on most villus sections
[c] +++ Several infected cells on all villus sections

Fig. 2. Villus from midgut, 23 hours p.i. The lateral epithelia contain numerous clefts or microcrypts, and many enterocytes have rounded apical margins. Compare with control (Fig. 3). H & E ×950 (total magnification)

Histology

The proximal intestine was unchanged throughout the experiment. No morphological alterations were seen in the midgut at 14 hours p.i. Many enterocytes in the ileum at 14 hours p.i. contained large ovoid vacuoles apical to the nucleus, although the villi were long and slender. Changes were first observed at 23 hours p.i. in the midgut. Here the villi were long and their lateral margins contained many clefts or microcrypts (Fig. 2), compared with control villi (Fig. 3). The enterocytes lining the lower one-third of the villi appeared normal, but those of the apical two-thirds had rounded margins and were cuboidal rather than columnar.

Fig. 3. Villi from midgut, 3 day-old control lamb. H & E $\times 380$

Many enterocytes contained large single basal vacuoles and multiple small apical vacuoles (Fig. 4). The apical vacuoles often impinged upon and indented the nucleus, which consequently appeared collapsed or pyknotic. Both apical and basal vacuoles contained pleomorphic Schiff-negative bodies which stained deep mahagony-red by Pollak's trichrome method. Similar intra-cytoplasmic bodies

were also seen in some enterocytes and subepithelial macrophages, usually close to the nucleus. These bodies were most clearly demonstrated in araldite sections (Fig. 5), and have been shown subsequently (Gray, E. W., in preparation) to contain arrays of astrovirus particles. The lamina propria of affected villi contained moderate numbers of macrophages with abundant cytoplasm. Goblet cell numbers were comparable to controls. The ileum was unaffected at this stage; single clear apical vacuoles were present in many of the enterocytes.

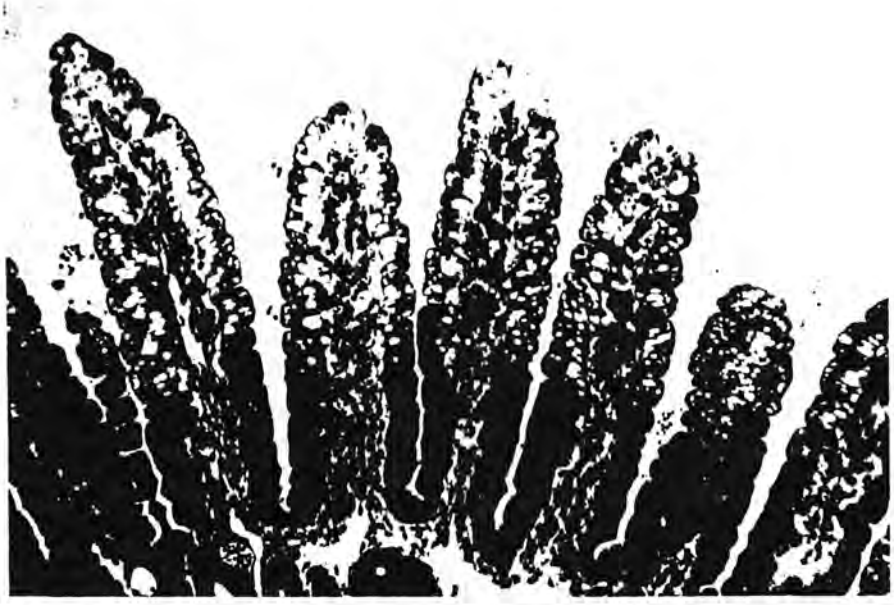

Fig. 4. Araldite section of villi, midgut site, 23 hours p.i. The basal portions of the villi are lined by normal columnar cells, while the apical two-thirds are lined by vacuolated cells with rounded margins. Microcrypts are numerous in distal villous margins. Giemsa ×140

By 38 hours p.i., villi in midgut and ileum were obviously shorter and more spatulate than those in equivalent control sites, or at earlier stages of the infection. At 45 hours p.i., villi in the midgut and ileum were short and blunt with crenated epithelium, and crypts which were elongated contained numerous mitotic figures. The lamina propria contained infiltrates of macrophages, lymphocytes and neutrophils, as well as eosinophils in similar numbers to the control lambs. None of the enterocytes in the midgut contained the multiple apical vacuoles and granular bodies seen at 23 hours p.i. However, single apical vacuoles were seen in the ileum of the infected lambs at 38 and 45 hours p.i. By 70 hours p.i. the villi in the midgut site were long and slender and indistinguishable from normal intestine, but those in the ileum were stunted and lined by a crenated, partly cuboidal epithelium. At 5 days p.i., however, all three intestinal sites were morphologically normal.

Large basal vacuoles were seen in midgut enterocytes of control lambs at 72 and 96 hours of age, but apical vacuoles were confined to the ileum of control lambs. These findings are in accord with those made in normal calves (8, 9) and piglets (2, 11, 12). The vacuolated cells may be absorptive with a marked pinocytotic capacity (11).

A few neutrophils were present in both caecal and colonic mucosa of the lambs killed 23 hours p.i., but not in any other lamb. No changes were found in any of the other tissues at any stage.

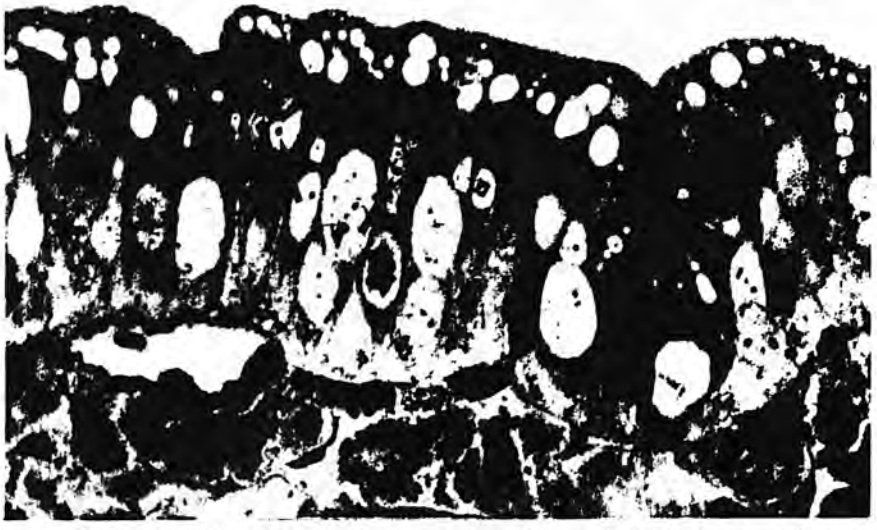

Fig. 5. Higher magnification of part of villus from Figure 4. Brush borders of enterocytes are intact. The apical cytoplasm contains numerous vacuoles which indent the nuclei. Dense bodies (arrows) can be seen in the cytoplasm or in small vacuoles close to the nucleus of some cells. Several of the large basal vacuoles contain coarse granular material. Large macrophages can be seen in the lamina propria. Giemsa. Oil immersion ×3800

Villus and Crypt Measurements

The villus heights and crypt depths at the three sampling sites in the five control lambs did not vary significantly with age. Normal measurements for villi and crypts at each site were therefore obtained by pooling observations for all five lambs. Measurements from individual infected lambs were compared with these normal values (Table 2).

The length of the villi in jejunum did not differ significantly from normal. Villus atrophy was observed at 38 and 45 hours p.i. in midgut, and from 38 to 120 hours p.i. in ileum. The crypts in all three sites showed a progressive elongation throughout the experiments. The most marked changes were observed in ileum, and are illustrated in Figure 6.

Table 2. *Histological measurements of villi and crypts*

Time of sample (hours p.i.)	Villi (μm, mean ± SE)			Crypts (μm, mean ± SE)		
	Jejunum	Midgut	Posterior ileum	Jejunum	Midgut	Posterior ileum
Controls	690 ± 14	613 ± 17	687 ± 15	124 ± 4	115 ± 3	105 ± 3
14	702 ± 23	581 ± 13	613 ± 20	159 ± 6[c]	139 ± 4[b]	122 ± 5[a]
23	771 ± 34[a]	589 ± 37	590 ± 17[a]	150 ± 8[b]	133 ± 8[a]	119 ± 8
38	683 ± 25	438 ± 17[c]	324 ± 24[c]	174 ± 5[c]	154 ± 5[c]	117 ± 5
45	732 ± 16	306 ± 21[c]	351 ± 20[c]	167 ± 8[c]	161 ± 10[c]	129 ± 6[b]
70	684 ± 20	540 ± 27	359 ± 10[c]	218 ± 7[c]	165 ± 8[c]	153 ± 4[c]
120	713 ± 24	804 ± 5[c]	439 ± 10[c]	225 ± 10[c]	176 ± 7[c]	172 ± 8[c]

Significance of deviation from control value

[a] $p < 0.05$
[b] $p < 0.01$
[c] $p < 0.001$

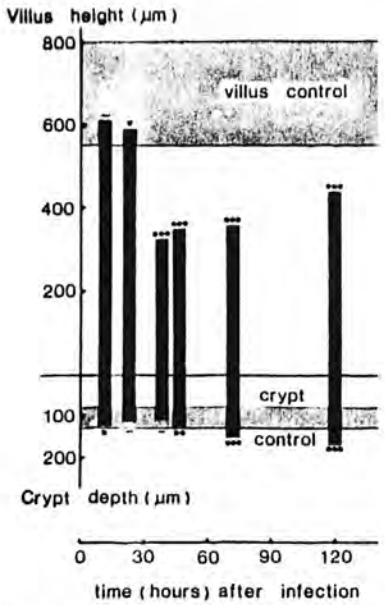

Fig. 6. Villus height and crypt depth in ileum. The crypt and villus controls are represented by a band for mean ± sd. The significance of the deviations from these control values are shown. — not significant, • $p < 0.05$, • • $p < 0.01$, • • • $p < 0.001$

Enzyme Analyses

Lactase levels in the 6 control lambs were 4.5 ± 0.5, 5.1 ± 0.6, and 2.4 ± 1.2 units/g tissue for the proximal, mid, and distal small intestinal sites respectively. In midgut of infected lambs, observations were consistently below those of the controls, falling to a minimum of 1.2 units/g tissue at 23 hours p.i. No consistent change was observed in lactase concentrations in proximal and distal sites.

Discussion

These experiments confirmed the ability of lamb astrovirus to multiply in the intestinal tract of gnotobiotic lambs, and to cause diarrhoea. The site of multiplication was shown by immunofluorescence and electron microscopy (GRAY, E. W., in preparation) to be the small intestine, and these same techniques failed to find evidence of astrovirus infection in any other site. Immunofluorescence showed less evidence of virus multiplication in jejunum than in other levels of small intestine, and this was reflected in the absence of histological change. Crypt hypertrophy was, however, as marked in jejunum as at more distal sites, but the stimulus for this is not known. Lamb rotaviruses have similarly been found to cause least damage in the jejunum (16).

Damage produced by astrovirus infection could be demonstrated by histopathology and estimations of lactase, and was consistently associated with a mild transient diarrhoea. A sequence of events with initial epithelial cell infection and destruction leading to partial villus atrophy, reclothing of the villi with relatively immature cells, and eventual healing of the lesion, can be postulated. This effect is similar to that of other viral infections of villus epithelial cells, particularly rotavirus (16, 18) and coronavirus (3, 10, 13) infections. In the lamb, astrovirus infection is at each stage less severe than rotavirus infection (16, 17), with fewer enterocytes infected, a lesser degree of villus atrophy and a milder clinical disease.

The astroviruses of lambs, calves, and man have not been shown to be serologically related by immunofluorescence. Further serological and biochemical studies are necessary to investigate the relationships between these viruses.

This study has confirmed that lamb astrovirus is a pathogen of the small intestine of lambs. However, the only information available on the epidemiology of any of the astroviruses is that antibody to bovine astrovirus was detected in cattle in 11 or 22 herds (19), so no attempt can be made as yet to define their role in causing diarrhoea under natural conditions.

Acknowledgments

The excellent technical assistance of Messrs J. Redmond and N. Inglis is gratefully acknowledged.

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Received December 14, 1978

Printed in Austria

J. gen. Virol. (1980), 49, 71–82
Printed in Great Britain

Ultrastructure of the Small Intestine in Astrovirus-infected Lambs

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(*Accepted* 31 *January* 1980)

SUMMARY

The ultrastructure of the small intestine of gnotobiotic lambs infected with lamb astrovirus was studied. The virus was observed from 14 to 38 h p.i. in mature columnar epithelial cells covering the apical two-thirds of villi. Crystalline arrays of virus particles with a centre to centre distance of approx. 29 nm were seen in the cytoplasm and virus particles were also observed in apical pits and tubules and in lysosomes. Macrophages containing virus particles in lysosome-like organelles were seen in the lamina propria. Virus particles were released by desquamated cells disintegrating in the gut lumen. Cuboidal cells lining villi appeared from 38 to 70 h p.i., and by 120 h p.i. the villi appeared normal.

INTRODUCTION

The name astrovirus has been suggested for viruses 28 to 30 nm in diam. with circular outlines and surface structure often arranged as a five or six pointed star (Madeley & Cosgrove, 1975). These morphologically distinctive viruses have been observed in the faeces of diarrhoeic children (Kurtz *et al.* 1977; Madeley *et al.* 1977; Ashley *et al.* 1978; Maass *et al.* 1978; Schnagl *et al.* 1978), lambs (Snodgrass & Gray, 1977) and calves (Woode & Bridger, 1978). Studies on the pathogenesis of astrovirus infection in lambs have shown the site of virus multiplication to be the small intestine (Snodgrass *et al.* 1979). No ultrastructural studies on the small intestine during astrovirus infection have been made although the appearance of human astrovirus in tissue culture has been reported (Kurtz *et al.* 1979). This paper describes the ultrastructure of the small intestine of astrovirus-infected lambs.

METHODS

Infection of lambs. Six 1 day-old gnotobiotic lambs were inoculated with intestinal contents from the third gnotobiotic lamb passage of lamb astrovirus (Snodgrass *et al.* 1979). One lamb was killed at each of the following times p.i.: 14, 23, 38, 45, 70 and 120 h. Six gnotobiotic lambs killed between 48 and 122 h of age were used as controls.

Preparation for electron microscopy. The lambs were anaesthetized and small pieces of intestine were taken from jejunum, mid-gut and ileum (Snodgrass *et al.* 1979). These were fixed by immediate immersion in 1 % glutaraldehyde in phosphate buffer (pH 7·4) at room temperature, diced into 1 mm^3, post-fixed in 1 % osmium tetroxide in phosphate buffer (pH 7·4), dehydrated in graded alcohols and embedded in Araldite. Location sections 1 μm thick were stained in 10 % Giemsa at 60 °C for 5 min. Ultrathin sections for electron microscopy were stained with saturated aqueous uranyl acetate followed by lead citrate and examined on a Siemens Elmiskop 1.

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RESULTS

Control lambs

The villi were covered for most of their length by mature columnar epithelial cells (Fig. 1). These cells had long glycocalyx-covered microvilli at their luminal margins and numerous apical pits in the luminal membrane (Pensaert *et al.* 1970), particularly in cells in the apical portion of villi. The cytoplasm contained wide terminal webs, apical tubular systems, numerous mitochondria, a few lysosomes and varying quantities of endoplasmic reticulum and glycogen. Autophagic vacuoles (Moon, 1976) were present in most cells in the apical two-thirds of the villi; large vacuoles tended to be basal while smaller vacuoles were widely distributed throughout the cell. The nucleus in these cells was irregular in outline and apical or medial in the cell, while in cells at the base of villi the nucleus was regular ovoid and basal. Cells at the base of villi were incompletely differentiated with short microvilli, no apical pits or autophagic vacuoles, poorly developed apical tubules and were deficient in glycogen and cell organelles. These cells also tended to be cuboidal in shape. A few degenerate epithelial cells were seen sloughing from extrusion zones at the tips of villi. Goblet cells were scattered throughout the villus epithelium and a few lymphocytes were seen in the lateral intercellular spaces betwen epithelial cells.

The lamina propria was mainly filled with small blood vessels but also contained a few lymphocytes, eosinophils and macrophages; the cytoplasm of the latter often contained numerous organelles resembling lysosomes. Plasma cells were rarely seen.

Infected lambs

Virus particles were found in mature villus epithelial cells in jejunum and ileum at 14 h p.i. and in all three gut sites at 23 and 38 h p.i. At 14 h p.i. infected cells were confined to the tips of villi but by 23 and 38 h p.i., virus particles were found in most epithelial cells covering the apical two-thirds of villi.

The cells shown in Fig. 2 are typical of infected cells at all times and sites. Electron-dense aggregates were observed in the cytoplasm of cells, and at a higher magnification (Fig. 3) these were seen to consist of circular hollow-cored particles in an amorphous matrix, often with apparently partly formed particles within the same matrix. Crystalline and quasi-crystalline arrays of solid or hollow-cored particles with a centre to centre distance of approx. 29 nm were also found either free in the cytoplasm, enclosed by a membrane or within secondary lysosomes (Fig. 4, 5 and 6). Virus particles in all of these forms could also be seen in autophagic vacuoles (Fig. 7). In a few infected cells, virus particles were seen in apical pits and tubules (Fig. 8). Virus particles lining the outer membrane of microvilli were observed at 23 and 38 h p.i. (Fig. 9). At these times degenerate epithelial cells, which were usually infected, were found sloughing from the apical portions of villi particularly in the mid-gut. The microvilli of these cells were disintegrating and they were deficient in ribosomes and cell organelles (Fig. 10). Release of virus particles from ruptured luminal margins of degenerating cells was not seen and virus particles were not seen in crypt cells.

By 38 h p.i. many of the columnar cells covering the villi had been replaced by cuboidal cells, particularly in the ileum (Fig. 11). These cells were similar in appearance to the immature incompletely differentiated cells seen at the base of villi in control lambs. They had short microvilli, round basal nuclei, few apical tubules and no apical pits or autophagic vacuoles. Cuboidal cells persisted at the tips of villi at 45 and 70 h p.i. but by 120 h p.i. the villi were indistinguishable from control villi. Infected cells were not seen after 38 h p.i.

From 23 to 70 h p.i. goblet cells were reduced in number and their contents were usually discharged, while increased numbers of lymphocytes were seen in the lateral intercellular spaces of epithelial cells. The lamina propria was increasingly infiltrated with lymphocytes, plasma cells and macrophages. The macrophages occasionally contained virus particles in organelles resembling lysosomes (Fig. 12).

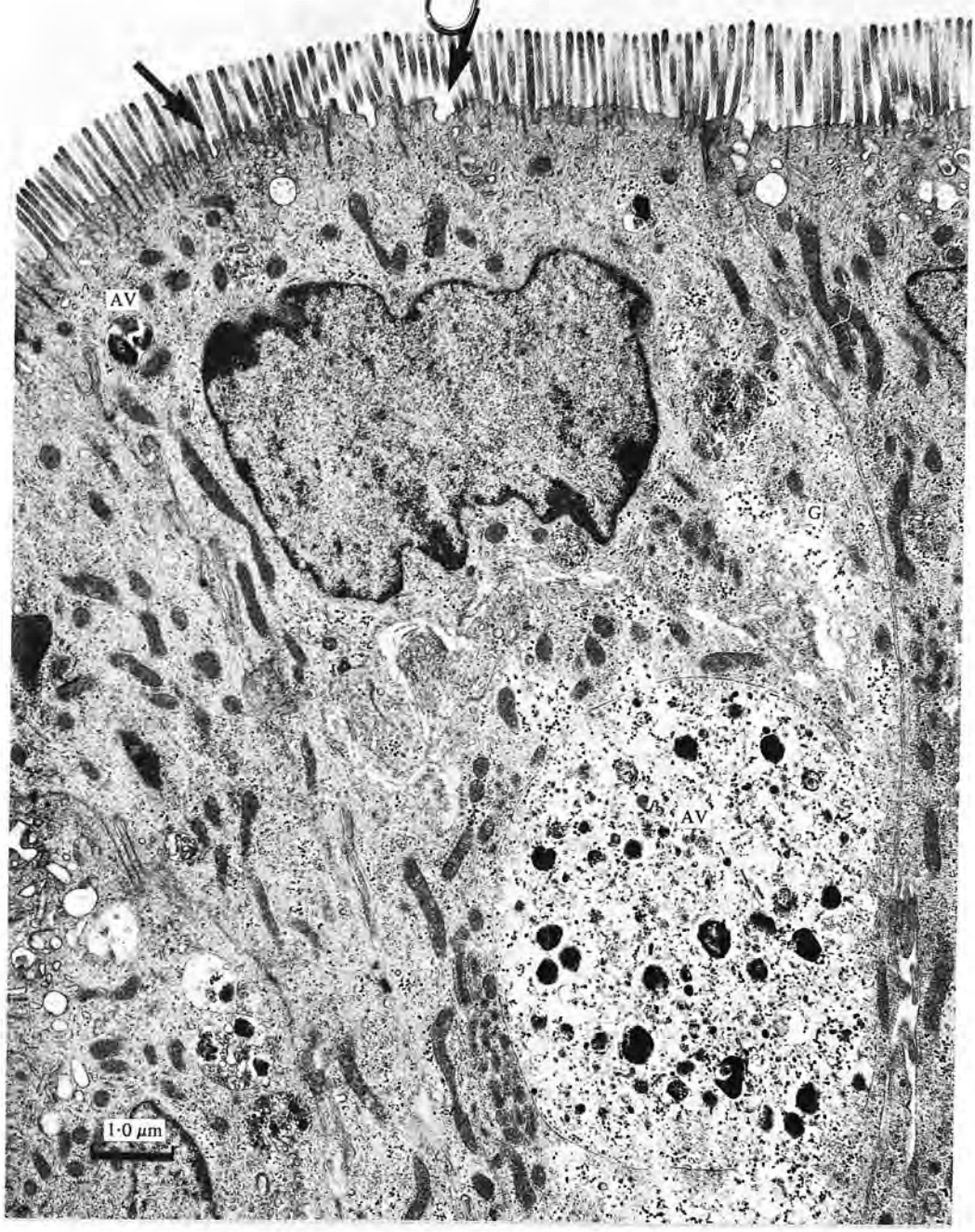


Fig. 1. Control mid-gut, 48 h old. A typical columnar villus epithelial cell showing autophagic vacuoles (AV), glycogen (G) and apical pits (arrows).

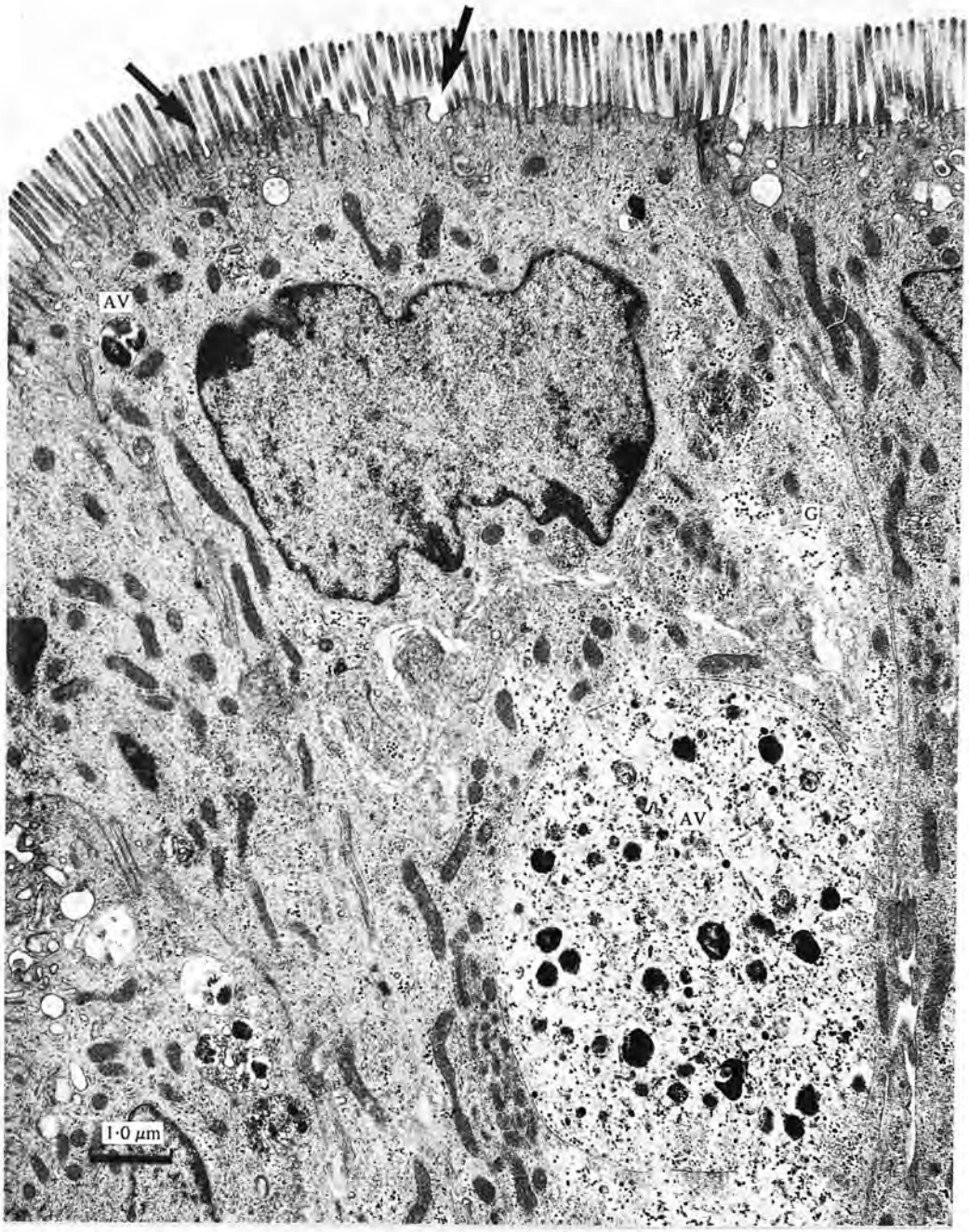


Fig. 1. Control mid-gut, 48 h old. A typical columnar villus epithelial cell showing autophagic vacuoles (AV), glycogen (G) and apical pits (arrows).

Fig. 2. Infected mid-gut, 23 h p.i. Villus epithelial cells with apical vacuoles and several virus aggregates (arrows). Autophagic vacuoles (AV) and apical tubules (AT) can also be seen.

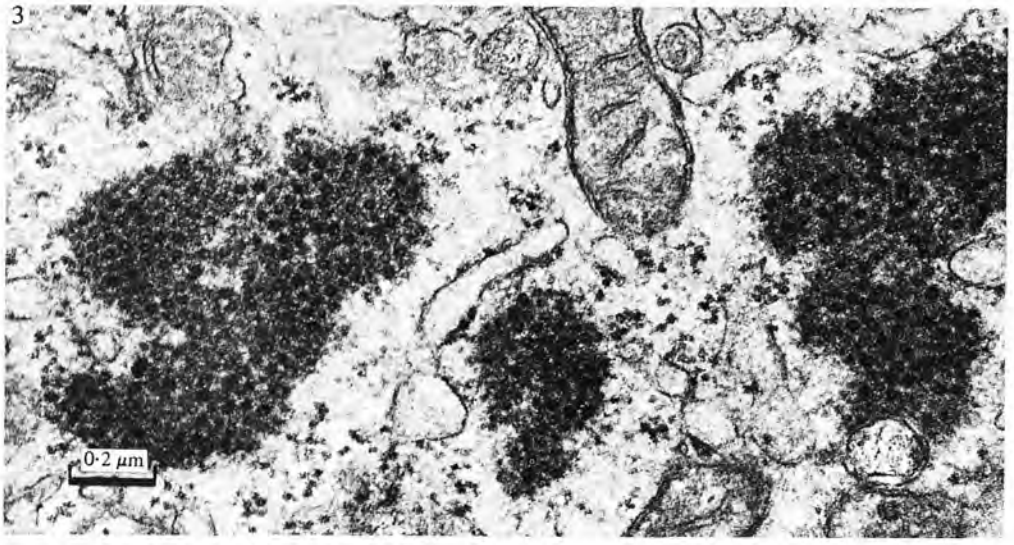
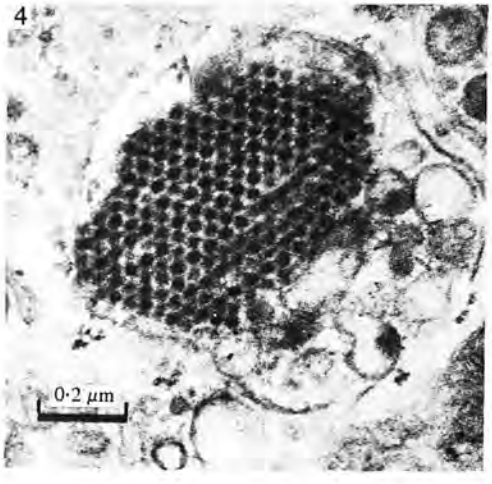


Fig. 3. Higher magnification of virus aggregates arrowed in Fig. 2, showing hollow-cored particles in an amorphous matrix.



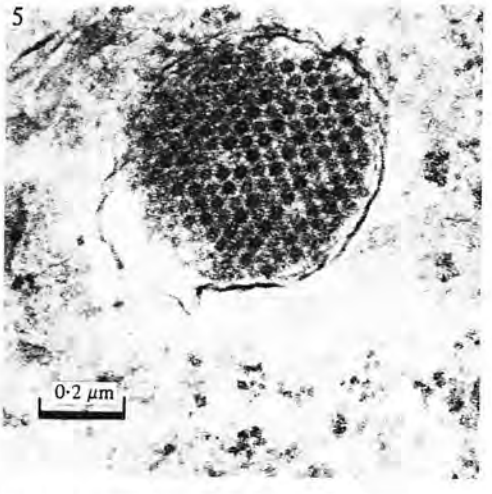


Fig. 4. Infected mid-gut, 23 h p.i. A crystalline array of solid particles enclosed by a membrane.

Fig. 5. Infected mid-gut, 23 h p.i. A crystalline array of hollow-cored particles enclosed by a membrane.

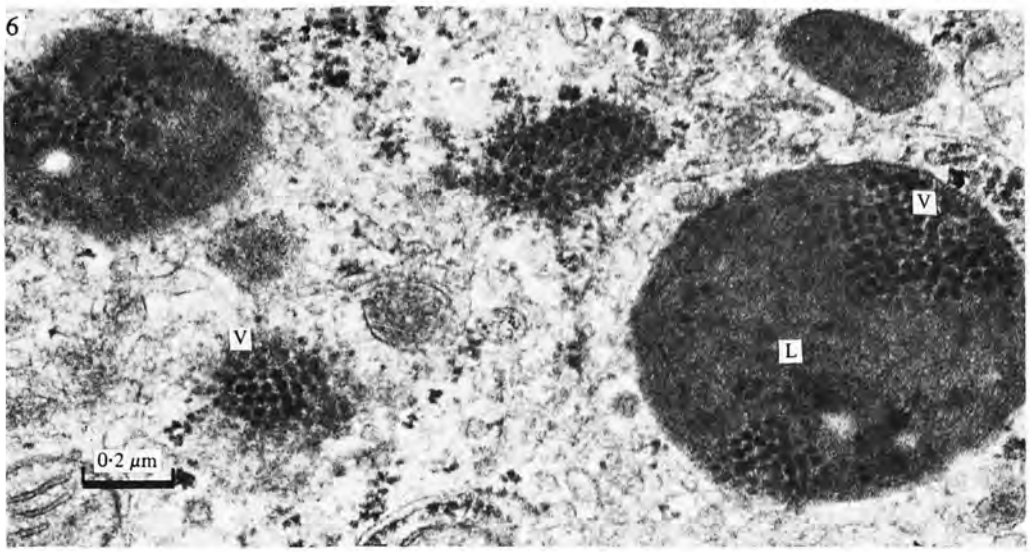


Fig. 6. Infected mid-gut, 38 h p.i. Virus particles (V) are shown in secondary lysosomes (L) and also free in the cytoplasm.

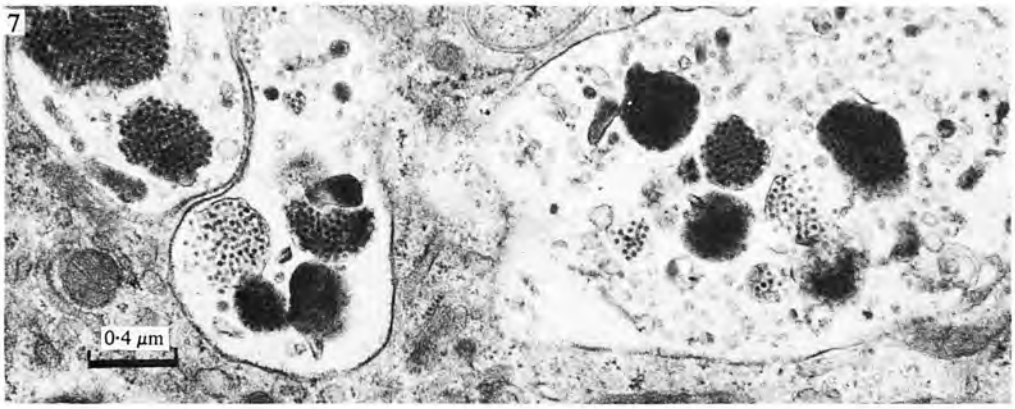


Fig. 7. Infected mid-gut, 23 h p.i., showing autophagic vacuoles in the cytoplasm of an epithelial cell. Virus particles can be seen in various formations within the vacuoles.

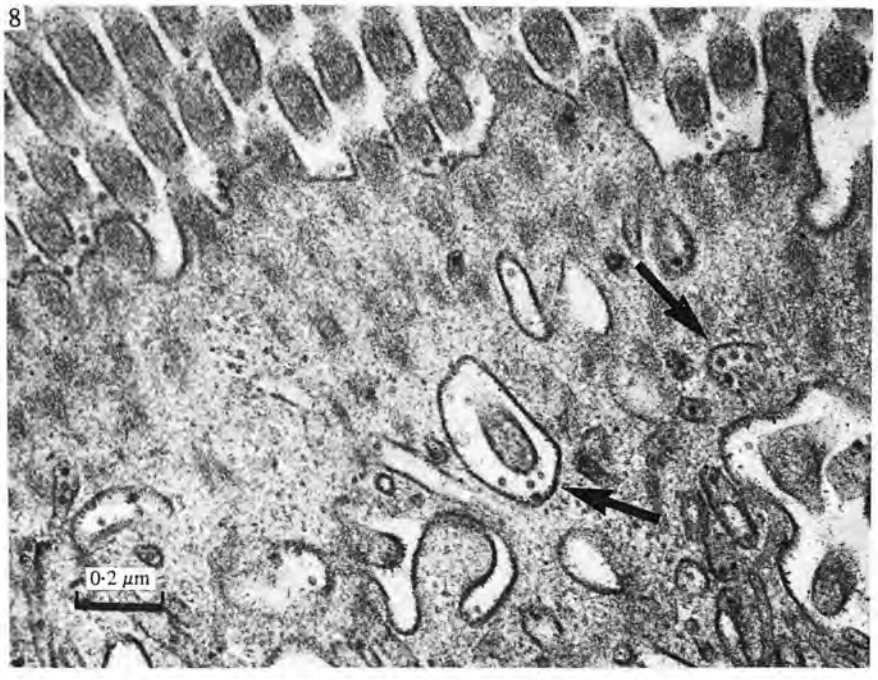


Fig. 8. Infected mid-gut, 23 h p.i. Virus particles can be seen in apical tubules (arrows) near the luminal margin.

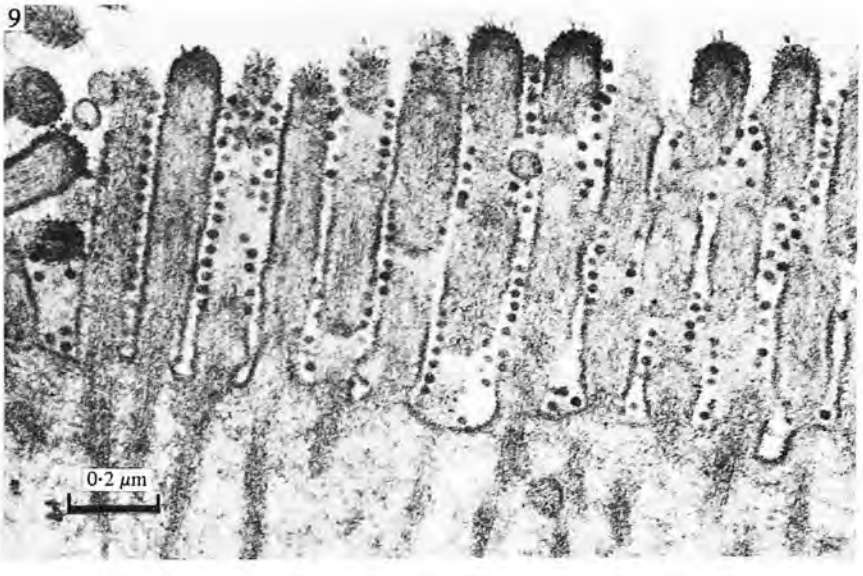


Fig. 9. Infected mid-gut, 38 h p.i. Virus particles are aligned along microvilli.

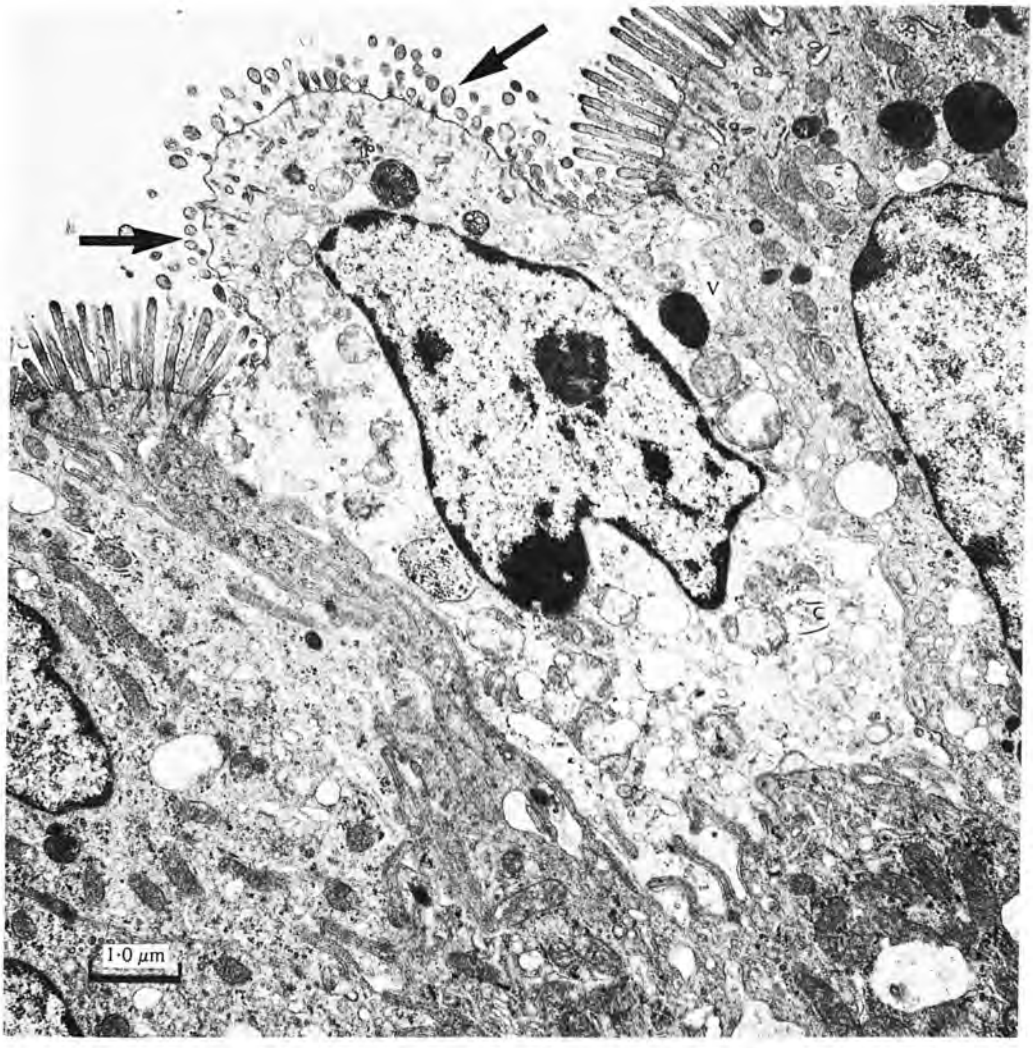


Fig. 10. Infected mid-gut, 38 h p.i. Degenerate epithelial cell sloughing into gut lumen. Note shedding microvilli (arrows) and virus particles (V) in secondary lysosome.

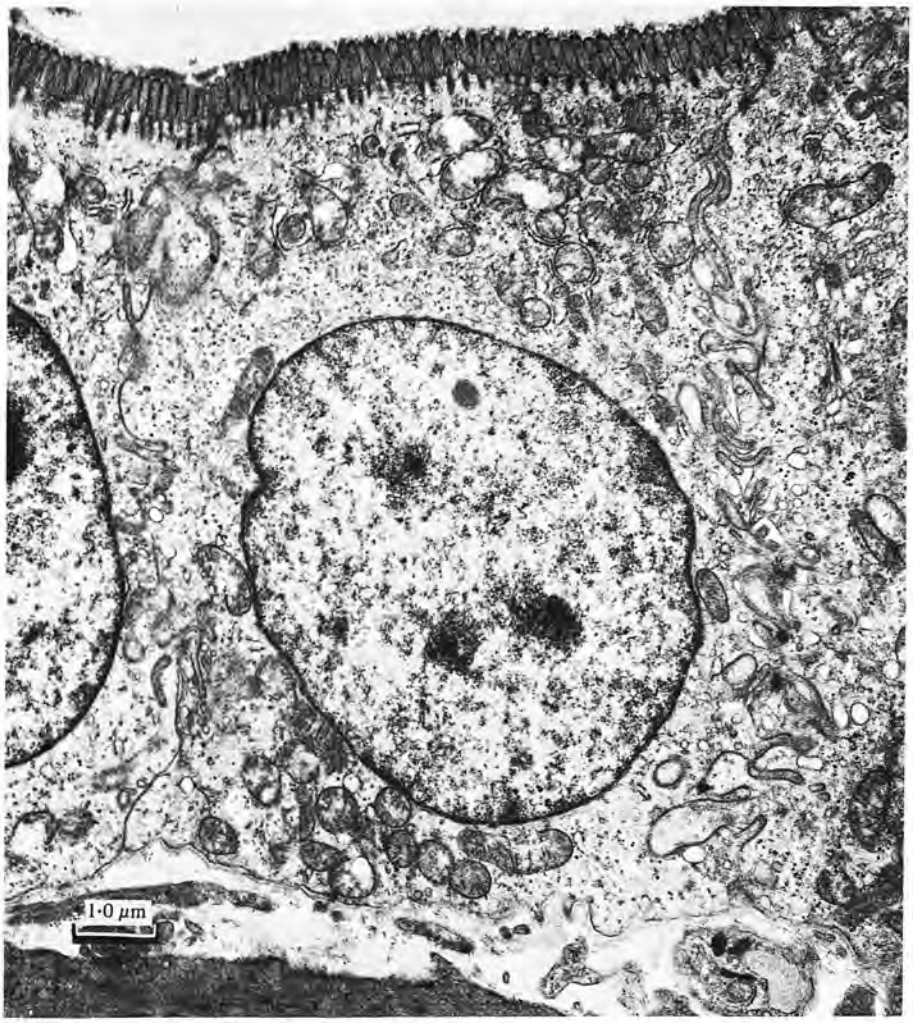


Fig. 11. Infected ileum, 38 h p.i. An immature cuboidal epithelial cell in the upper portion of a villus. Note short microvilli and lack of autophagic vacuoles, apical pits and tubules.

Fig. 12. Infected mid-gut, 23 h p.i. Cells infiltrating the lamina propria, including a plasma cell (P), lymphocyte (L) and macrophage (M). The inset shows virus particles in the lysosome-like organelle arrowed, at higher magnification.

DISCUSSION

Previous histological and immunofluorescent studies of lamb astrovirus infection showed that the sole target site of virus multiplication was the small intestine (Snodgrass *et al.* 1979). Ultrastructural studies, therefore, were concentrated on this site and have now confirmed that the site of virus multiplication is the mature villus epithelial cell. The numbers of infected cells increased from 14 to 38 h p.i. and infected cells were often observed sloughing into the gut lumen at 23 and 38 h p.i. These sloughed cells were replaced by immature cuboidal cells which persisted at villus tips until 70 h p.i. By 120 h p.i. the villi appeared normal.

Kurtz *et al.* (1979) have briefly described the appearance of human astrovirus in the cytoplasm of HEK cells. This virus was seen as arrays of dense round particles with a centre to centre spacing of 28 nm. Thus, human astrovirus in tissue culture cells resembles lamb astrovirus in villus epithelial cells, but insufficient detail of the human astrovirus was given by these authors for further comparisons to be made. Furthermore, the human astrovirus produced in tissue culture cells is apparently non-infective.

Apical pits in cells of the small intestine have been shown to be the route of entry of transmissible gastroenteritis virus (TGE) in pigs (Pensaert *et al.* 1970) and an adenovirus and an adeno-associated virus in rats (Worthington & Graney, 1972*a*). Absorptive cells of the ileum and jejunum are capable of withdrawing intact adenovirus particles from the intestinal lumen and transporting them to degradative organelles (Worthington & Graney, 1972*b*). In this study astrovirus particles were found in apical pits and tubules and this was considered to be the route of entry into epithelial cells. Immature replacement epithelial cells lacked apical pits and tubules and therefore were not infected. From these tubules virus particles presumably pass into the cytoplasm where they may enter secondary lysosomes. The role of lysosomes in the multiplication of astroviruses is not clear. Virus particles lining microvilli were observed at 23 and 38 h p.i. as observed with TGE virus in pigs by Pensaert *et al.* (1970) who suggested that maturation of TGE virus occurred at this site. Astroviruses were never seen passing through the membranes of microvilli in either direction, however, and the precise reason for their presence at this site could not be ascertained. Virus particles apparently within microvilli were thought to be a sectioning artefact arising from the small size of the virus (28 nm) within a relatively thick section (50 nm approx.), resulting in distorted spatial relationships. TGE virus is released into the gut lumen through ruptured luminal membranes (Pensaert *et al.* 1970) but this was not observed with astroviruses. The presence of considerable numbers of astrovirus particles, particularly at 38 h p.i., lining microvilli was therefore presumed to be the result of disintegrating desquamated cells releasing virus.

Astroviruses are RNA viruses with genomes similar to those of picornaviruses (A. J. Herring, personal communication). The ultrastructural studies reported here and studies reported previously (Snodgrass & Gray, 1977) have shown that lamb astrovirus is similar in size to some picornaviruses and also multiplies in the cytoplasm. However, further physicochemical characterization is required before the astrovirus can be assigned to any of the virus families.

The authors would like to acknowledge Pat Gray for invaluable technical assistance and Alan Inglis for preparing the photographs.

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(*Received* 28 *November* 1979)

J. gen. Virol. (1981), **53**, 47–55
Printed in Great Britain

Purification and Characterization of Ovine Astrovirus

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(*Accepted 1 October 1980*)

SUMMARY

Astrovirus, purified in aggregated form from epithelial cells of the small intestinal villi of infected gnotobiotic lambs, was shown to have a single-stranded RNA genome with an S value of 34 and poly(A) tract. Only two major capsid polypeptides were detected with similar mol. wt. of approx. $3{\cdot}3 \times 10^4$.

INTRODUCTION

Astrovirus was first described by Madeley & Cosgrove (1975) as a small round virus, 28 to 30 nm in diam., commonly observed in the diarrhoeic stools of babies with gastroenteritis. It was recognized as a novel virus species since it showed a distinctive morphology and the name astrovirus was proposed because some of the particles had a five- or six-pointed stellate configuration. Astroviruses have since been described associated with diarrhoea in lambs (Snodgrass & Gray, 1977) and calves (Woode & Bridger, 1978).

Attempts to grow all three astroviruses in cell culture have failed although it has been possible by immunofluorescent techniques to demonstrate limited replication of both human and bovine viruses on the first passage (Lee & Kurtz, 77; Woode & Bridger, 1978). In addition, the ultrastructure of human astrovirus in cultured cells has been described (Kurtz *et al.*, 1979).

Lamb astrovirus has been shown to replicate in the columnar absorptive epithelial cells of the small intestine and to produce symptoms of mild diarrhoea in gnotobiotic lambs (Snodgrass *et al.*, 1979; Gray *et al.*, 1980). In this communication we report the purification of ovine astrovirus from intestinal cells. Characterization of this virus has shown that the RNA genome resembles that of the picornaviruses and caliciviruses but that the polypeptide composition is unlike that of either of these groups.

METHODS

Growth of virus. Gnotobiotic lambs were infected by the oral route with virus-infected gut contents at 24 h after birth. Animals were killed from 24 to 48 h post-inoculation (p.i.), the gut removed and contents extruded. The small intestine was cut into 25-cm sections which were then opened lengthwise, pinned out and the mucosal surface lightly scraped with a microscope slide. Examination of these intestinal scrapings by optical microscopy showed that they contained large numbers of detached villi. Each lamb yielded from 5 to 10 ml of such material.

Purification of virus

Method 1. Intestinal scrapings were suspended in 20 ml phosphate-buffered saline (PBS, 0·008 M-sodium phosphate buffer pH 7·2, 0·14 M-NaCl) containing 1% (w/v) SDS (PBS/SDS) per ml of scrapings. The mixture was then thoroughly blended with a glass–teflon homogenizer and centrifuged at 560 *g* for 5 min. The supernatant was removed and centrifuged at 95000 *g* for 1 h at 20 °C and the pellets resuspended, with the aid of a homogenizer, in 15 ml PBS. Aggregation of the virus occurred and the resulting precipitate of

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virus was pelleted and washed twice with PBS by centrifugation at 560 *g* for 10 min. Such preparations are referred to below as crude virus or crude virus pellets.

Method 2. Five to 7 ml of intestinal scrapings were suspended in 100 ml PBS containing 1% (w/v) Triton X-100 (BDH) (PBS/TX-100) and disrupted with seven strokes of a glass–teflon homogenizer. The mixture was cleared at 1250 *g* for 10 min and the pellets washed with 20 ml PBS/TX-100. Supernatants were pooled and centrifuged at 10000 *g* for 30 min at room temperature. The resulting large pellets were resuspended in 30 ml PBS/SDS with a homogenizer, recentrifuged at 95000 *g* for 1 h at 20 °C and finally washed twice in PBS as for the final pellets in method 1.

Most results presented in this paper were obtained with virus preparations from three lambs. These were: B216 killed at 24 h p.i. and processed by method 1, C254 killed 48 h p.i. and processed by method 2 and C259 killed 24 h p.i. and processed by method 2.

CsCl gradient centrifugation. Virus pellets from the methods above were resuspended i PBS containing 1% (w/v) Sarcosyl (Ciba-Geigy, N.Y., U.S.A.) and 20 mM-2-mercaptoethanol and incubated at 37 °C for 30 min followed by a 3 min treatment in a bath-type sonicator. This suspension was then loaded on to a 10 to 46% CsCl gradient consisting of five layers with concentrations of 10, 19, 28, 37 and 46% (w/w) CsCl in PBS, Sarcosyl, mercaptoethanol buffer and centrifuged for 17 h at 114000 *g* in an SW40Ti rotor (Beckman) at 20 °C. In one experiment, a preformed linear 10 to 46% (w/v) CsCl gradient was used and the centrifugation time was 2·5 h. The gradients were fractionated using an ISCO model 185 fractionator and u.v. monitor by upward displacement with Maxidens (Nyegaard & Co., Oslo, Norway).

Nucleic acid preparation. Virus pellets were resuspended in 50 mM tris-HCl pH 8·3 containing 2% (w/v) SDS and 0·1 M-NaCl and extracted twice with 0·6 vol. phenol–cresol mixture (Parish & Kirby, 1966) and 0·4 vol. chloroform. The final aqueous phase was precipitated with 2 vol. ethanol at −20 °C overnight. Ribonuclease A (Sigma) digestion was performed in 0·15 M-NaCl, 0·015 M-sodium citrate pH 7 (1 × SSC).

Sucrose gradient centrifugation of RNA. Virus RNA was analysed on 10 to 30% (w/v) sucrose gradients in 50 mM-tris-HCl pH 7 containing 0·1 M-NaCl, 0·001 M-EDTA and 0·5% (w/v) SDS. Gradients of 12·5 ml were centrifuged for 4·5 h at 202000 *g* and 20 °C in an SW40Ti rotor (Beckman) and fractionated as for the CsCl gradients. Sedimentation coefficients were calculated relative to 18S and 28S ribosomal RNA standards centrifuged i parallel gradients by the method of Martin & Ames (1961).

Polyacrylamid gel electrophoresis of RNA. This was performed as described by Loening (1967) using 2·3 and 2·6% gels. Gels were scanned with a Gilford 252 spectrophotometer fitted with a gel transport system and amounts of virus RNA calculated from the peak areas by comparison with known quantities of ribosomal RNA. Gels were stained overnight with 0·001% toluidine blue (Sigma) after prior washing for 5 h with H_2O; under these conditions double-stranded nucleic acids stain pink and single-stranded species stain blue (Bevan *et al.*, 1973).

Reverse transcriptase assay for poly(A) RNA. To 1 μg of sucrose gradient-purified virus RNA was added 50 μl reaction mixture containing 40 mM-tris-HCl pH 8·3, 50 mM-NaCl, 5 mM-$MgCl_2$, 15 μg/ml bentonite, 1 mM dATP, dTTP and dCTP, 10 μCi ^{3}H-dGTP (13·2 Ci/mmol), 12·5 units of avian myeloblastosis virus reverse transcriptase and 0·25 μg oligo(dT) (Boehringer, Mannheim). RNA and oligo(dT) were omitted in control incubations and poly(rC).oligo(dG) (P.-L. Biochemicals, Milwaukee, Wis., U.S.A.) was added as a positive control. After 30 min incubation at 37 °C the reaction was stopped by dilution with 200 μl of a 0·02% solution of bovine serum albumin (BSA) and cooling to 4 °C. Two 120 μl amounts were taken and precipitated with 10% TCA, harvested on to Whatman GFC glass fibre filters, washed, dried and counted in toluene-based scintillation fluid.

Table 1. *Effect of various treatments on astrovirus aggregates in gut contents as judged by observation with the electron microscope*

Reagent	Concentration	Treatment	Result
Chloroform	50% (v/v)	Emulsification	No disaggregation
Arcton	50% (v/v)	Emulsification	No disaggregation
NaCl	2 M	Incubation at ambient temperature	No disaggregation
CsCl	2·6 M	Incubation at ambient temperature	No disaggregation
Triton X-100	1% (w/v)	Incubation at ambient temperature	No disaggregation
SDS	1% (w/v)	Incubation at ambient temperature	No disaggregation
Sarcosyl	1% (w/v)	Incubation at ambient temperature	No disaggregation
Trypsin	2·5 mg/ml	Incubation at 37 °C for 30 min	No disaggregation
EDTA	1 mM	Incubation at ambient temperature	No disaggregation
EDTA	1 mM	Ultrasonication for 5 min	No disaggregation
Urea	3 M	Incubation at 37 °C for 30 min	Very few particles survived treatment
Mercaptoethanol plus Sarcosyl	20 mM 1% (w/v)	Incubation at 37 °C for 30 min with 3 min mild sonication	Partial disaggregation

Hybridization of virus RNA with ^{3}H-poly(U). The method used was that of Bishop *et al.* (1974).

Polyacrylamide gel analysis of virus polypeptides. This was performed as described by Weber & Osborn (1969) and Laemmli (1970), using phosphorylase A, BSA, catalase, ovalbumin, aldolase and chymotrypsin (Boehringer, Mannheim) as mol. wt. standards. Gels were stained with 0·25% (w/v) Coomassie blue after fixation in 20% (w/v) sulphosalicylic acid.

Electron microscopy of astrovirus. Preparations of purified astrovirus were dried on to formvar/carbon-coated grids, stained with 1% phosphotungstic acid pH 7 for 30 s and examined in a Siemens Elmiskop 1A electron microscope.

RESULTS

Purification of virus

All the procedures described were monitored by electron microscopy which was the only method available to detect virus. Initial attempts to purify the virus from infected faeces or gut contents utilized the fluorocarbon extraction and CsCl density-gradient techniques which successfully purified rotavirus (Todd & McNulty, 1976). This approach failed for astrovirus since the majority of the virus particles were aggregated, either with other particles or with cell debris, and were consequently lost in the initial low-speed centrifugations. A variety of treatments were then investigated to attempt to disaggregate the virions in gut contents. The results are summarized in Table 1; none of the treatments led to the production of discrete virions but incubation with mercaptoethanol and Sarcosyl did prevent aggregation with debris. These experiments showed that the particles were stable to a range of reagents including ionic detergents.

The discovery that virus multiplied in the epithelial cells of the small intestine (Gray *et al.*, 1980) led us to investigate this tissue as a source of virus. Virus was isolated from cells scraped from the mucosal surface by exploiting its stability to treatment with the ionic detergent SDS. However, whilst simple disruption of the cells with this detergent was successful, it was found to be preferable to carry out the initial lysis with Triton X-100 as this avoided the high viscosity of the homogenate caused by the release of DNA from chromatin (see methods 1 and 2 above).

Neither method resulted in virus pellets which could be disaggregated in buffer irrespective of the presence of detergent. The crude preparation was thus an aggregate of virus which could be sedimented by low-speed centrifugation. Aggregates of several thousand particles

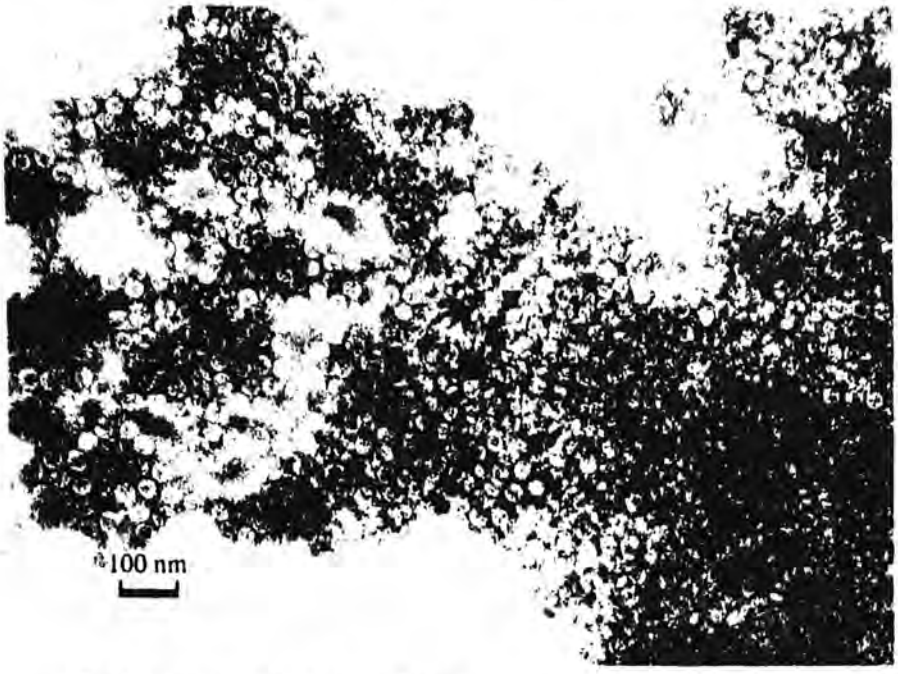

Fig. 1. Astrovirus. A crude preparation of aggregated particles produced by method 1.

were seen with the electron microscope as illustrated in Fig. 1. Particle morphology was poorly preserved and very few virions showed the typical stellate surface configuration. Only occasional pieces of contaminating cell debris were observed in such preparations.

CsCl density-gradient centrifugation

The absorbance profile of a crude astrovirus preparation centrifuged overnight on a preformed gradient of CsCl is shown in Fig. 2, together with the amount of virus RNA in the relevant fractions. The virus nucleic acid was identified and estimated by pelleting tl individual fractions at high speed followed by phenol extraction and analysis of the nucleic acid on polyacrylamide gels (see below). The absorbance profile shows two peaks at densities of 1·365 and 1·39. The denser peak was due to a very sharp band which was clearly visible and could be seen to consist of aggregated virus. Virus was also present in the fractions containing the upper peak but the particles were observed to be single. The bulk of the virus RNA (see below) was found in the fraction which contained the band of aggregated virus. Similar gradient profiles were obtained with overnight centrifugation on a stepped preformed gradient, or with short centrifugation (2·5 h) on a linear continuous CsCl gradient. The buoyant densities observed with crudely purified intracellular virus were consistent with those obtained in an attempt to purify virus from gut contents using self-generating gradients of CsCl in which virus was mainly present in fractions with densities from 1·38 to 1·40 g/ml.

Analysis of astrovirus nucleic acid

Nucleic acid preparations obtained by phenol extractions of both crude and CsCl-purified virus were analysed by sucrose density-gradient centrifugation and by gel electrophoresis (Fig. 3 and 4). All preparations contained a nucleic acid species with an estimated sedimentation coefficient of 34S (Fig. 3). This species was identified as single-stranded RNA

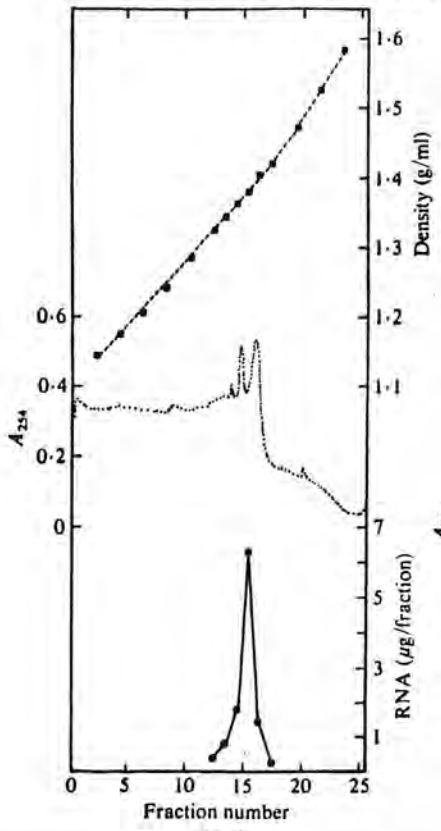

Fig. 2

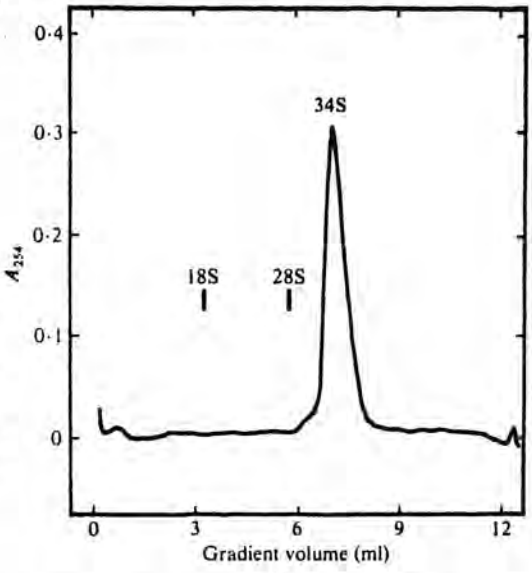

Fig. 3

Fig. 2. CsCl density-gradient centrifugation of crude astrovirus from lamb C259. ■---■, Density of individual fractions; ·····, absorbance profile at 254 nm; ●——●, virus RNA content of individual fractions.

Fig. 3. Sucrose gradient analysis of nucleic acid from a crude astrovirus preparation from lamb C259 produced by method 2. The positions of 18S and 28S ribosomal RNA standards in a parallel gradient are shown.

by its sensitivity to digestion with 1 μg/ml ribonuclease A in 1 × SSC buffer (Fig. 4) and by the broad band it produced in polyacrylamide gels, which stained blue with toluidine blue. It had an apparent mol. wt. of $2{\cdot}7 \times 10^6$, as judged by aqueous gel electrophoresis relative to ribosomal RNA standards. The 34S RNA was the only species detected in nucleic acid prepared from crude virus purified by method 2 and CsCl gradient-purified virus; in a preparation of crude virus produced by method 1 some DNA contamination was evident from the gel profiles. The association of the 34S RNA with the virus particles was confirmed by the CsCl gradient data (Fig. 2).

Results obtained with reverse transcriptase and hybridization with ^{3}H-poly(U) both indicated that the 34S astrovirus RNA contained a poly(A) tract. The results shown in Table 2 were obtained when 34S RNA was incubated with reverse transcriptase, nucleotide triphosphates and oligo(dT) primer; ^{3}H-dGTP was used as the label so that only DNA synthesis on a heteropolymeric RNA template was detected. DNA synthesis occurred only in the presence of the oligo(dT) primer. One result obtained by ^{3}H-poly(U) hybridization is presented in Fig. 5. The method was found to estimate the poly(A) standards quantitatively.

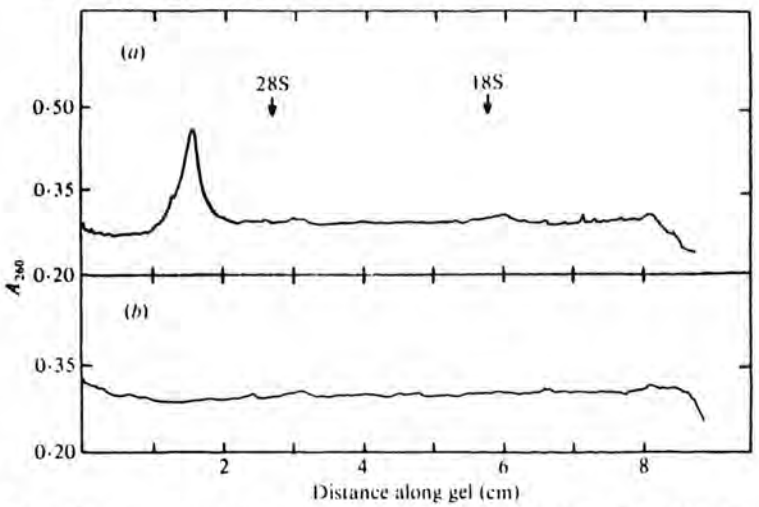

Fig. 4. Polyacrylamide gel electrophoresis of astrovirus RNA from lamb B216. (*a*) Absorbance profile of a gel loaded with virus RNA; the positions of the peaks of 18S and 28S ribosomal RNA in a parallel gel are indicated. (*b*) Profile of a gel loaded with virus RNA which had been incubated with 1 μg/ml ribonuclease A.

Table 2. *Incorporation of ^{3}H-dGTP into DNA by reverse transcriptase using astrovirus RNA as a template as measured by acid-insoluble radioactivity*

Additions to reaction mixture			
Oligo(dT) (0·5 μg)	RNA (0·5 μg)	Poly(rC).oligo(dT) (0·25 μg)	Ct/min
–	–	–	326
+		–	327
+	+ (B216)	–	92214
+	+ (C259)	–	115707
–	+ (B216)	–	2594
–	+ (C259)	–	1446
–	–	+	882235

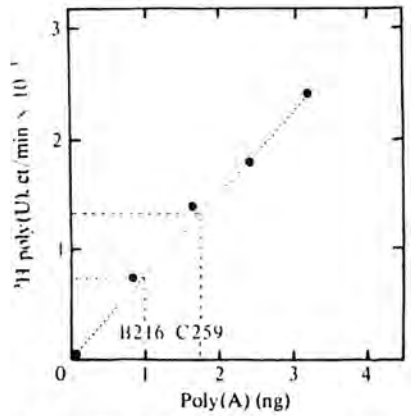

Fig. 5. Estimation of the poly(A) content of astrovirus RNA by ^{3}H-poly(U) hybridization. ●·····●, Ct/min in ribonuclease-resistant ^{3}H-poly(U) found with the poly(A) standards. Dotted lines to the ordinate and abscissa indicate the counts found when astrovirus RNAs from two virus preparations (1 μg of each) were hybridized under identical conditions and the equivalent amounts of poly(A).

Fig. 6. Slab-gel electrophoresis of astrovirus polypeptides from lamb C254. Tracks 1 and 2, mol. wt. standards (see Methods); tracks 3 and 4, astrovirus polypeptides.

The levels of hybridization with 34S RNA were all positive but were variable; from three such experiments estimates of the size of the poly(A) tract varied from 7 to 30 residues, with a mean of 14.

Polyacrylamide gel electrophoresis of astrovirus polypeptides

Gel analysis of polypeptides from crude and CsCl gradient-purified virus was performed in both continuous and discontinuous buffer systems using a range of gel concentrations. Optimum resolution was achieved with an 11% gel and discontinuous buffer system as shown in Fig. 6. Two polypeptide species, present in roughly equimolar amounts, were seen when gradient-purified virus was analysed. The mol. wt. of these two closely migrating polypeptides was estimated as approx. $3{\cdot}3 \times 10^4$. A similar value was obtained using the continuous buffer system but the two polypeptides were not resolved. When crude virus preparations were analysed, up to three additional faint bands were seen on the gels.

DISCUSSION

The purification of astrovirus is complicated by aggregation which is a property of both human and ovine astrovirus. Bridges between the particles which appear to be projections from the virus surface can be seen with the electron microscope (Madeley & Cosgrove, 1975; Snodgrass & Gray, 1977) and clearly the formation of the crystalline arrays described by Gray *et al.* (1980) requires inter-particle bonds. Virus in the gut lumen is found both in aggregates of various size and as single particles, which suggests that the large aggregates and

crystalline arrays seen in the cytoplasm of infected cells are broken down either during or after release from infected cells. The purification method described above isolates these intracellular aggregates directly and the non-dispersible nature of the crude virus pellets and their appearance in the electron microscope suggests that further aggregation takes place during the purification.

It has been suggested that the formation of aggregates has a function in the preservation of infectious virus in the environment outside the cell but the property of aggregation is usually only found under certain well-defined conditions of ionic strength and pH (Galdiero, 1979). Astrovirus aggregates appear to be extremely stable and certainly cannot be dispersed merely by raising the salt concentration; a detailed study of the effects of pH on aggregation has not been carried out but preliminary experiments in which the pH was lowered to 4 did not appear to produce disaggregation (A. J. Herring, unpublished results). Now that preparations of purified aggregated astrovirus can be produced, studies of the nature of the inter-partic bonds should be facilitated.

The interpretation of the data from the CsCl gradient is also complicated by aggregation of the particles. The absorbance profile shows two clear peaks, the denser of which consisted of aggregated particles so that its area in the absorbance profile does not measure the amount of virus present. As RNA analysis showed, the bulk of the virus was clearly in this aggregated peak. Heterogeneity of buoyant density has also been reported for the calicivirus which causes vesicular exanthema of swine, the two peaks of virus occurring at the same densities as those found for astrovirus but with the major peak at 1·36 g/ml. This effect was attributed to differences in solvation and Cs^+ ion binding (Oglesby *et al.*, 1971). The effect of aggregation on these properties may explain the two peaks of astrovirus.

The nucleic acid analysis showed the genome of astrovirus to be a single-stranded RNA molecule very similar in its sedimentation and electrophoretic behaviour to those found in the picornavirus and calicivirus groups (Newman *et al.*, 1973; Kerr & Martin, 1972). Astrovirus RNA also resembles the genome of these viruses in the possession of a short poly(A) tract, the estimated size of which resembled that reported for encephalomyocarditis virus RNA (Giron *et al.*, 1976; Emtage *et al.*, 1976). The variability of the ^{3}H-poly(U) hybridization results (Table 2) was most probably due to the acid lability of poly(U) described by Williams & Klett (1978).

The results of the polypeptide analysis showed that astrovirus is not a calicivirus sin members of this group have a single characteristic major polypeptide with a mol. wt. of abou twice that found for astrovirus (Bachrach & Hess, 1973; Burroughs & Brown, 1974). However, the results do not allow the virus to be classified as a picornavirus since members of this group possess four major structural polypeptides (Cooper *et al.*, 1978). It is possible that other structural proteins have been lost due to the harsh treatments used in purification. However, picornaviruses are able to withstand treatment with 1% SDS (Kerr & Martin, 1972; Talbot *et al.*, 1973).

Judged on the present evidence, the astroviruses must be considered a separate group with a polypeptide composition intermediate between that of the picornavirus and calicivirus. A final decision on their classification will have to await further elucidation of their structure.

We wish to thank Dr J. Gruber of the Office of Program Resources and Logistics, N.I.H., and Dr J. W. Beard of Life Sciences Inc., Florida for their kind gift of purified reverse transcriptase.

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(*Received 2 July 1980*)

Chapter 17

Astroviruses in Diarrhea of Young Animals and Children

DAVID R. SNODGRASS

I. INTRODUCTION

The name "astrovirus" was first used by Madeley and Cosgrove in 1975 to describe a "small round virus" observed by electron microscopy in stools from babies in Scotland with gastroenteritis. They believed that this virus was morphologically distinct from various other small viruses observed in stools. The virus particles were circular in outline, with a surface configuration of a five- or six-pointed star; hence the suggested name "astrovirus." Since this initial description, viruses fulfilling these morphological criteria have also been reported

COMPARATIVE DIAGNOSIS
OF VIRAL DISEASES, VOL. IV

ISBN 0-12-429704-8

homogenate with a 23-gauge needle tipped with plastic

Pairs of mice from each group were killed daily on days 3 to 8 p.i. and were examined for cryptosporidial

from diarrheic children in England (Kurtz *et al.*, 1977; Ashley *et al.*, 1978) and Australia (Schnagl *et al.*, 1978). Viruses indistinguishable in appearance have also been identified in feces from calves (Woode and Bridger, 1978) and lambs (Snodgrass and Gray, 1977). Confirmation that the astroviruses were in fact vertebrate viruses, and not bacteriophages or cell debris, was first obtained by transmission of lamb astrovirus to gnotobiotic lambs (Snodgrass and Gray, 1977).

There is no official approval for the name "astrovirus" from the International Committee on Taxonomy of Viruses, and indeed insufficient information as yet exists to enable these viruses to be classified. However, it has found favor with those actively working with neonatal diarrhea in man and animals, and so is used in this chapter in preference to more clumsy alternatives.

II. DESCRIPTION OF ASTROVIRUSES

A. Morphology

No differences have been detected in the appearance of astroviruses in feces of children, lambs, or calves. After negative contrast staining with either 1% potassium phosphotungstic acid (pH 7.0) or 1% ammonium molybdate (pH 5.3), the viruses appear circular in outline and 28–30 nm in diameter (Madeley and Cosgrove, 1975; Snodgrass and Gray, 1977; Woode and Bridger, 1978). Empty particles, which have a rim 3–4 nm thick, are rarely seen (Woode and Bridger, 1978). A five- or six-pointed stellate configuration is apparent on some particles, being seen on about 10% of the human astroviruses stained with potassium phosphotungstate (Madeley and Cosgrove, 1975). However, after staining with ammonium molybdate, some surface structure can be seen on nearly all particles, with recognizable star shapes on many of them (Fig. 1). The lamb astrovirus is usually observed in feces as large aggregates, with occasional bridging structures between particles. The human astrovirus can occur in quasi-crystalline arrays, with a 6.5-nm gap between adjacent particles (Madeley and Cosgrove, 1975).

Examination of thin sections of infected lamb small intestine shows astroviruses in villus epithelial cells (Fig. 2) and occasionally in subepithelial macrophages. The viruses are seen in viroplasm (Fig. 3) or crystalline arrays (Fig. 4) within the cytoplasm of infected cells, and occasionally within membranes or in vacuoles (Fig. 5). Hollow-cored particles are sometimes evident. The mean diameter of 103 of these particles is 24.8 ± 0.6 nm.

B. Physicochemical Properties

Experiments to characterize lamb astrovirus are incomplete, but some information has already been obtained (A. J. Herring, personal communication). The

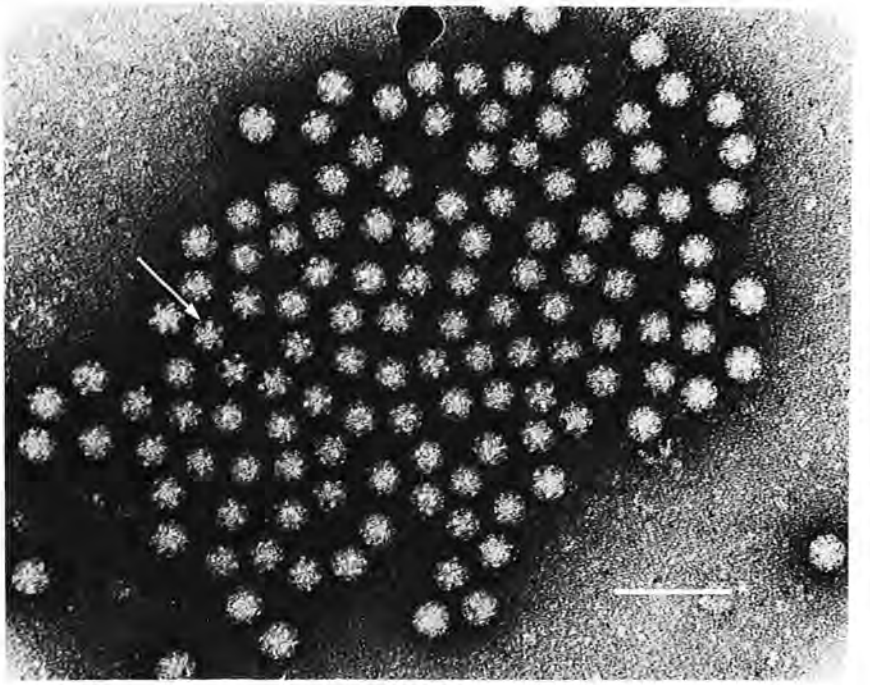

Fig. 1. Lamb astrovirus particles in intestinal content of experimentally infected gnotobiotic lamb. The arrow indicates a group of particles showing a star-like surface structure. The bar represents 100 nm. Stained with ammonium molybdate. (Reproduced by permission of *Archives of Virology*.)

work is limited by the fact that the astrovirus is present in feces and intestinal contents largely in aggregates, which cannot be disrupted by ultrasound, and also by the fact that the only method of virus assay is electron microscopic visualization. The best preparations have been made from scrapings of small intestinal mucosa of infected lambs. In cesium chloride density gradients, virus particles are seen most often at a density of 1.38 gm/ml. In the single experiment so far in which a substantial preparation of astrovirus has been achieved, RNA extraction yielded a single-stranded RNA molecule with a molecular weight 2.7×10^6 and with a sedimentation coefficient of 35 S on sucrose gradients. This molecule contains a poly(A) tract.

The virus is stable to the following range of treatments as determined by electron microscopy: organic solvents—chloroform, Arcton 113; high salt concentrations—2 *M* NaCl, 2 *M* CsCl; detergents—1% SDS, 1% Sarcosyl, 1% Triton X100; enzymes—trypsin. The virus is sensitive to 3 *M* urea in PBS after 30 minutes at 37°C.

homogenate with a 23-gauge needle tipped with plastic

days 3 to 8 p.i. and were examined for cryptosporidial

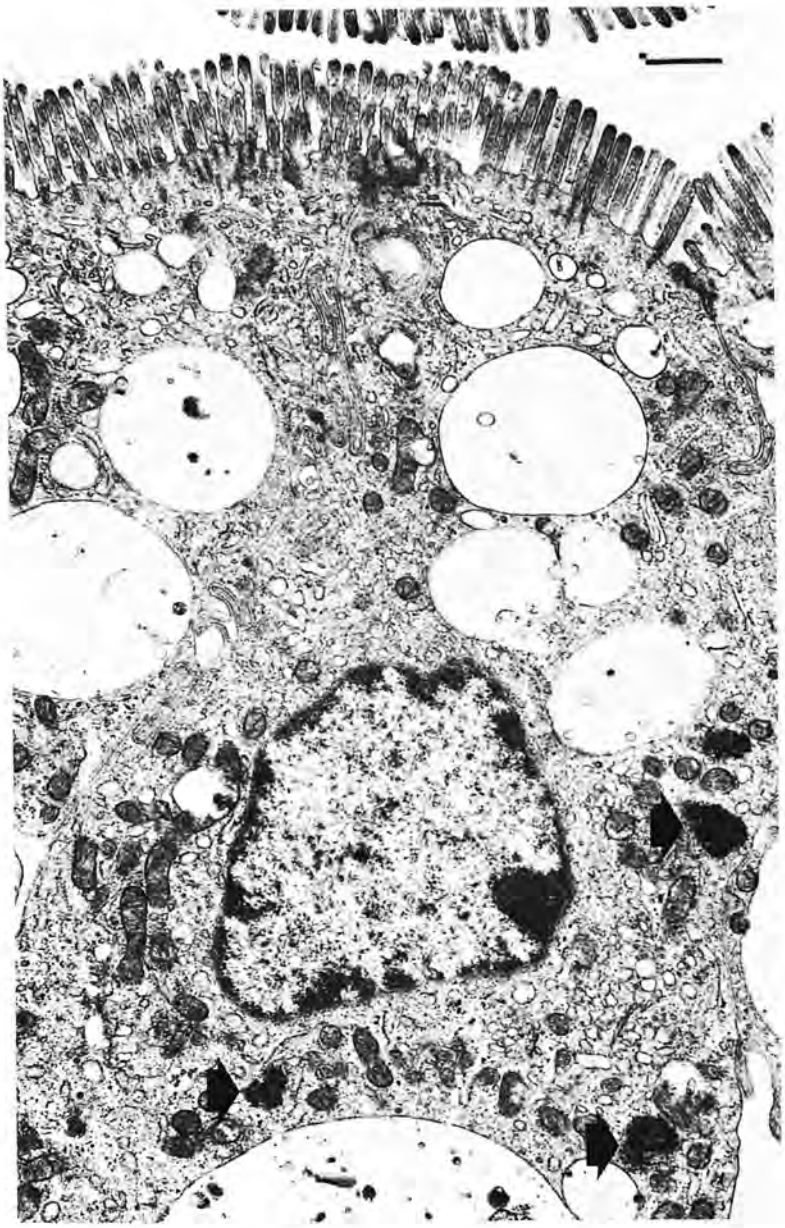

Fig. 2. Villus epithelial cell containing astrovirus (arrowed). Bar respresents 1 μm. Figs. 2, 3, 4, and 5 stained with lead citrate and uranyl acetate.

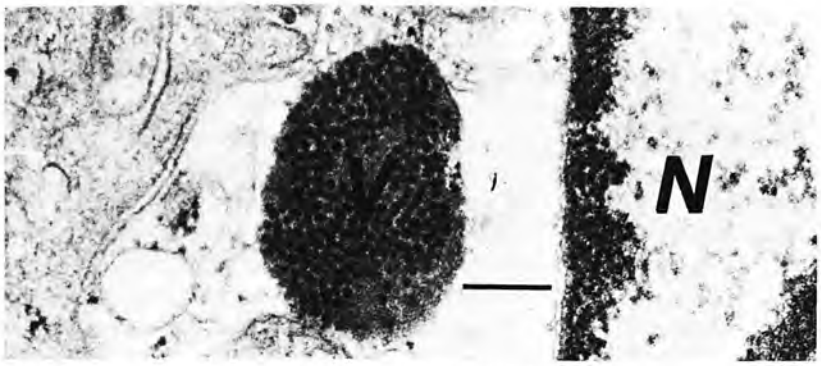

Fig. 3. Viroplasm (V) adjacent to nucleus (N) of epithelial cell. The bar represents 200 nm.

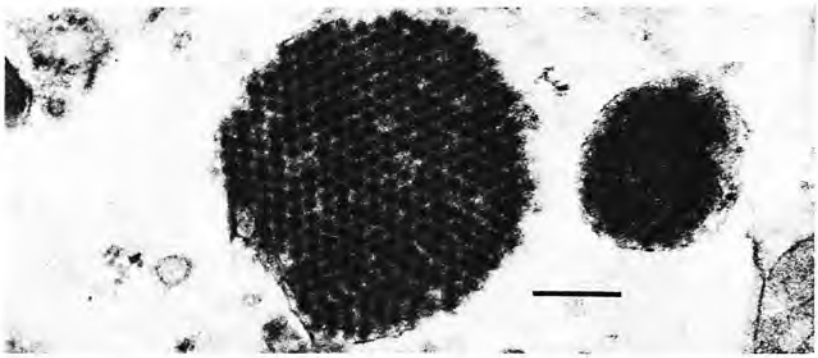

Fig. 4. Crystalline array of astrovirus in epithelial cell. The bar represents 200 nm.

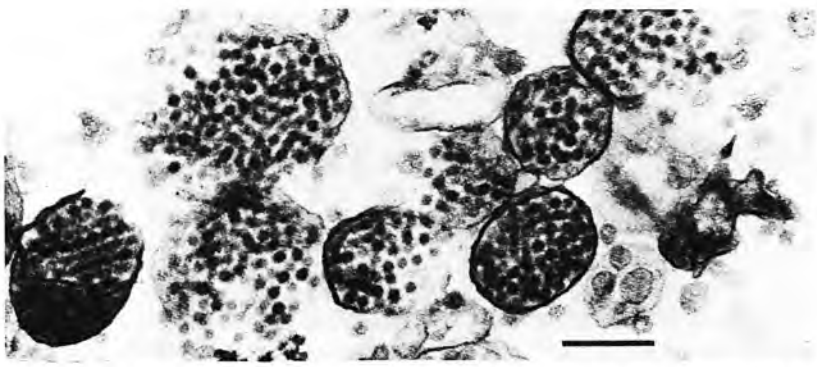

Fig. 5. Membrane-bound astrovirus in cytoplasmic vacuole. The bar represents 200 nm.

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III. COMPARATIVE BIOLOGY AND PATHOGENESIS

A. Pathogenesis in Animals

Astrovirus infections have been produced experimentally in gnotobiotic lambs (Snodgrass and Gray, 1977) and calves (Woode and Bridger, 1978) by oral inoculation of bacteria-free fecal filtrates (0.22 μm) of intestinal contents containing astrovirus. However, pathogenesis has so far been studied only in lambs (Snodgrass *et al.*, 1979). A mild diarrhea occurred in lambs after an incubation

Fig. 6. Section of lamb small intestine stained by immunofluorescence with lamb antiserum to lamb astrovirus. Virus antigen is present in epithelial cells on villi. (Reproduced by permission of *Archives of Virology*.)

period of about 48 hours and lasted 1–2 days. Virus excretion in feces was detected at a time approximately coincident with the onset of diarrhea. By use of immunofluorescence on tissue sections, the virus was found to multiply only in the small intestine, predominantly in the epithelial cells on the distal parts of the villi (Fig. 6). There was also evidence of occasional infection of subepithelial cells. The greatest numbers of infected enterocytes were found during the incubation period, and only scattered fluorescent cells were seen after diarrhea had commenced.

Histological damage was confined to the middle and posterior small intestine. Villi were shorter and more spatulate than in control lambs and lined with a crenated epithelium, which contained some cuboidal cells. The villous lamina propria contained infiltrates of macrophages, lymphocytes, and neutrophils. Occasional intracytoplasmic inclusions could be seen, which were shown by electron microscopy to consist of large numbers of astrovirus particles. Measurements of villus height and crypt depth throughout the infection confirmed the partial villus atrophy, villus height at the time of onset of diarrhea being only half of that in control lambs. Crypt hypertrophy developed subsequent to the villus atrophy and was still marked after clinical recovery.

Electron microscopic examination of thin sections of small intestine confirmed that some villus epithelial cells were cuboidal and also demonstrated infected necrotic epithelial cells sloughing into the gut lumen (Fig. 7).

Obviously no detailed pathogenetic mechanism can be postulated on this limited evidence. However, the demonstration of epithelial cell infection and subsequent destruction is similar to that of other viral enterites, particularly rotaviral and coronaviral infections (Pensaert *et al.*, 1970; Mebus *et al.*, 1975; Snodgrass *et al.*, 1977), and such damage is likely to interfere with the normal digestive and absorptive functions of these cells.

B. Pathogenesis in Man

Studies with human astrovirus infections in adult volunteers have shown the ability of the virus to infect the gastrointestinal tract after oral inoculation with bacteria-free fecal filtrates (Kurtz *et al.*, 1979). Symptoms produced were mild, with diarrhea and vomiting in only 1 of 17 adults and mild constitutional symptoms in several of the others.

C. Tissue Culture

Attempts to adapt lamb astrovirus to ovine embryo kidney cell cultures by routine techniques have failed. Application of techniques that have been found helpful for rotavirus propagation, i.e., trypsin treatment of both virus and cultures (Theil *et al.*, 1978), centrifugation of cell cultures after inoculation (Banat-

homogenate with a 23-gauge needle tipped with plastic

days 3 to 8 p.i. and were examined for cryptosporidial

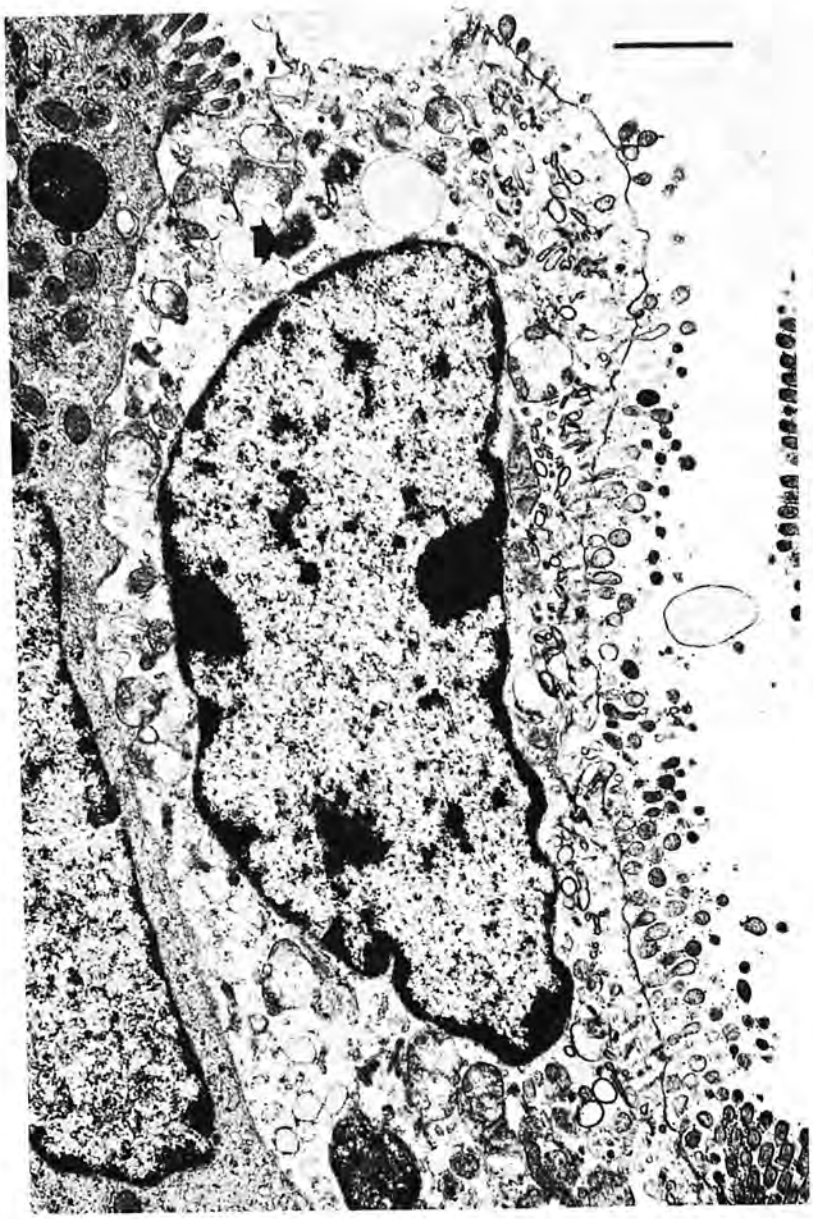

Fig. 7. Necrotic epithelial cell containing astrovirus (arrow) in the process of sloughing. Numerous astrovirus particles can also be seen among the remains of the microvilli. The bar represents 1 μm. Stained with lead citrate and uranyl acetate.

vala *et al.*, 1975), and immunofluorescence at daily intervals, has likewise failed to show any evidence of virus replication. Attempted propagation of calf and human astroviruses has been similarly unsuccessful, with the exception that both these viruses have been able to infect cell cultures on primary passage only, as demonstrated by immunofluorescence (Lee and Kurtz, 1977; Woode and Bridger, 1978).

IV. SEROLOGY

Techniques for estimating antibody to astrovirus are limited, due to current inability to propagate the virus *in vitro*. Immunofluorescence tests have been carried out, using as antigen either lamb astrovirus in gut sections (Snodgrass *et al.*, 1979) or calf or human astrovirus in nonproductive cell culture infections (Woode and Bridger, 1978; Kurtz *et al.*, 1979). An immune electron microscopy technique has also been used for estimation of human astrovirus antibodies (Kurtz *et al.*, 1977).

The relationship between the three astroviruses so far recognized has been examined by immunofluorescence. Using each astrovirus in turn as antigen, specific fluorescence has been detected only with the homologous antiserum. Similarly, no cross reaction has been observed between human astrovirus and rotavirus, Norwalk agent, or the W agent (Kurtz *et al.*, 1979).

V. EPIDEMIOLOGY

Preliminary antibody surveys suggest that astrovirus infections are common in cattle (Woode and Bridger, 1978) and man (Kurtz *et al.*, 1979), and astroviruses were detected in 26 of 183 sporadic cases of diarrhea in babies (Madeley *et al.*, 1977). Thus, as the viruses appear quite widespread and capable of producing disease, they probably make some contribution to the overall occurrence of neonatal diarrhea. Accurate definition of the extent of this contribution is not possible at present.

VI. LABORATORY DIAGNOSIS

A. Demonstration of Virus in Feces

To demonstrate astrovirus in lamb feces, we make a 20% suspension of feces in distilled water. After the larger debris is allowed to settle, a drop of the supernatant fluid is transferred to a carbon collodion-coated grid, stained with 1%

potassium phosphotungstic acid (pH. 7.0), and examined in the electron microscope at a magnification of 40,000. We have compared this examination of crude stool suspension with results obtained by clarification and ultracentrifugation and found both to be equally sensitive. Techniques for detecting astroviruses in calves and children have usually employed concentration procedures (Kurtz *et al.*, 1977; Madeley *et al.*, 1977; Ashley *et al.*, 1978; Woode and Bridger, 1978). The particles have to be distinguished from other small viruses present in feces, particularly calici-like viruses (Madeley and Cosgrove, 1976). The stellate configuration of astroviruses is usually distinct from the large surface hollows of the calicivirus, and in particular the calicivirus "Star of David" configuration with a central hollow is unmistakable. However, where very few particles are present, it may be impossible to be certain of their identity.

The ability of the human astrovirus to produce nonproductive infections in cell culture that can be detected by immunofluorescence can also be utilized as a diagnostic method provided a specific immune astrovirus serum without antibody to rotavirus is available.

The future application of modern techniques suitable for examining large numbers of samples, such as enzyme-linked immunosorbent assay, will depend on the production of high-titer specific antisera, while as yet only convalescent sera are available.

B. Demonstration of Virus in Gut Sections

Immunofluorescent staining of cryostat sections of small intestine obtained at biopsy or necropsy can be used to demonstrate astrovirus antigen in epithelial cells. However, the absence of infected cells is not a conclusive negative finding, as the highest rate of cell infection is present during the incubation period (Snodgrass *et al.*, 1979).

C. Demonstration of a Serological Response

Serological diagnosis of astrovirus infections has been used only in human infections, and the methods available are either immunofluorescence or immune electron microscopy (Kurtz *et al.*, 1977, 1979; Ashley *et al.*, 1978).

ACKNOWLEDGMENTS

I would like to thank my colleagues K. W. Angus, E. W. Gray, and A. J. Herring for their cooperation throughout this work.

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Pairs of mice from each group were killed daily on days 3 to 8 p.i. and were examined for cryptosporidial

INFECTION AND IMMUNITY, Nov. 1982, p. 471–475
0019-9567/82/110471-05$02.00/0

Vol. 38, No. 2

Experimental Cryptosporidiosis in Laboratory Mice

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Received 22 April 1982/Accepted 14 July 1982

Eight strains of laboratory mice were susceptible to subclinical infections with *Cryptosporidium* sp. at 1 to 4 days of age, but only a transient infection could be established at 21 days of age or older. Immunosuppression of 21-day-old mice failed to render them more susceptible to infection. Laboratory storage conditions for *Cryptosporidium* sp. were investigated by titration in 1- to 4-day-old mice. Storage by freezing with a variety of cryoprotectants was unsuccessful, but storage at 4°C in phosphate-buffered saline or 2.5% potassium dichromate was possible for 4 to 6 months.

Cryptosporidium sp. is a member of the family *Cryptosporididae*, suborder *Eimeriorina*, but it differs from most other enteric coccidia in that the organisms are smaller (diameter, 3 to 4 μm) and only invade the microvillous border of gut enterocytes.

Cryptosporidium sp. infections were described first in tamed wild mice (16, 17) and subsequently in C57 mice (7). Infections associated with diarrhea have also been reported in a variety of animals and in humans (2, 5, 10, 13, 18, 20, 21, 23, 24, 28).

Studies in guinea pigs (26, 27) suggested that *Cryptosporidium* sp. is species specific, but recently, subclinical infections were recorded in 1-day-old specific-pathogen-free (SPF) suckling mice and rats inoculated with feces containing *Cryptosporidium* sp. taken from calves, lambs, and humans (19).

This paper describes the use of laboratory mice as models for the study of several aspects of *Cryptosporidium* sp. infections: susceptibility of mouse strains, age-related susceptibility, infections in immunosuppressed mice, and the effect of multiple passage through mice on the pathogenicity of the organisms for lambs. Storage in laboratory media was also investigated.

MATERIALS AND METHODS

Animals. All animals were bred as SPF at the Moredun Research Institute and were maintained in plastic isolators. Two random-bred strains (Schneider Swiss White and Porton) and six inbred strains (CBA, CBA Nude, C57 Black, BALB/c, Porton [derived from the random Porton stock], and Hairless [HR/HR-ADR]) were used. Inbred Porton mice were used except where stated otherwise. Two gnotobiotic lambs were also used.

Administration of inocula to mice. Mice were inoculated orally with 0.1 ml of fecal suspension or gut homogenate with a 23-gauge needle tipped with plastic tubing. The dose was increased to 0.2 ml for mice 21 days or more in age.

Preparation of inocula. *Cryptosporidium* sp., isolated from the feces of a diarrheic calf, was passaged in SPF rats, gnotobiotic lambs (22), and SPF mice and was used to prepare inocula 1 through 4 (Fig. 1).

All inocula were prepared as 20% (wt/vol) homogenates of feces (inoculum 1) or whole gut (inocula 2 through 4) in 5% bovine serum albumin (BSA). The fecal preparation used in the storage experiment contained a calf *Cryptosporidium* sp. isolate that had been passaged four times in gnotobiotic lambs (D. R. Snodgrass, unpublished data) and had an initial starting 50% mouse infective dose of 3.08 ($\log_{10}$).

Susceptibility of mouse strains. Two litters each of eight strains of mice (Table 1) were inoculated between 1 and 4 days of age. Mice were killed and examined daily from 2 to 7 days post inoculation (p.i.) for evidence of infection.

Age-related susceptibility. Four litters of suckling mice were inoculated with inoculum 2 at 4 days of age, and 24 weaned mice were inoculated with inoculum 3 at 21 days of age. Two mice from each group were killed and examined for infection on 11 occasions from 2 to 16 days p.i. In addition, mice of the Hairless, Swiss White, and CBA Nude strains (14 mice from each strain) were inoculated at 21 to 42 days of age with inoculum 1, and 1 mouse from each strain was examined daily for evidence of infection for 14 days p.i.

Immunosuppressed mice. A total of 24 mice 21 days old were treated with cyclophosphamide at doses of 70 mg/kg, with two doses given intraperitoneally 7 days apart (1). Of these mice, 12 were inoculated with inoculum 4 at 2 days after the second cyclophosphamide injection. The immunosuppresive activity of cyclosphosphamide was evaluated by assaying the serological response to the injection of louping-ill virus vaccine, as was done before with respect to TAB vaccine (1). Louping-ill virus vaccine (Moredun type vaccine, Wellcome) was given to all cyclophosphamide-treated mice and to 12 control mice.

Pairs of mice from each group were killed daily on days 3 to 8 p.i. and were examined for cryptosporidial

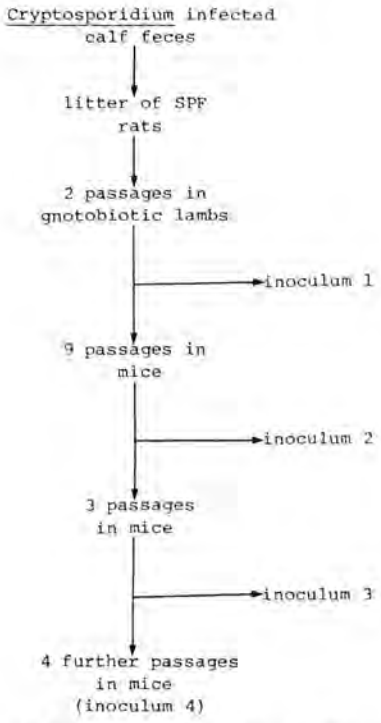

FIG. 1. Sequential passage of a calf *Cryptosporidium* sp. isolate.

infection. Serum antibody titers to louping-ill virus were measured by the hemagglutination inhibition test (4).

Effect of multiple passage on the pathogenicity for lambs. Two gnotobiotic lambs 2 days old were orally inoculated with 5 ml of the 12th mouse passage of *Cryptosporidium* sp. (inoculum 3). Under terminal anesthesia, tissue was taken from the intestine of one lamb at 5 days p.i. and from that of the other lamb at 11 days p.i. for histological examination (25).

Storage of *Cryptosporidium* sp. The effects of freezing and freeze-drying on inoculum 3 in the presence of cryopreservatives under various conditions (Table 2) were investigated. All cryopreservatives were made up in phosphate-buffered saline (PBS), pH 7.2 (Table 2), and were mixed with equal volumes of inoculum 3.

In addition, the stability of cryptosporidia stored for 6 months at 4°C in distilled water, PBS, 5% BSA, and 2.5% potassium dichromate was studied. Cryptosporidia were also stored in PBS at 15 to 20 and at 37°C.

To titrate the stored preparations, half-$\log_{10}$ dilutions were made in PBS, and one litter (average, six mice) of 1- to 4-day-old mice was inoculated with each dilution. Mice were killed 7 days p.i., and cecal contents were examined for the presence of cryptosporidia. The 50% mouse infective dose was calculated by the Reed-Muench method (14).

Detection of *Cryptosporidium* sp. Smears of cecal contents were made on glass microscope slides, air-dried, fixed in methanol for 2 min, and then were immersed in Giemsa stain (45 ml of distilled water, 2.5 ml of Giemsa solution [BDH], 3.0 ml of methanol, 0.2 ml of 1.5% sodium carbonate solution) for 1 h. After staining, smears were carefully rinsed in tap water, air-dried, and examined by light microscopy (×1,000) with oil immersion.

Segments from duodena, terminal ilea, and ceca were taken into 10% buffered formol saline or Carnoy fixative. Paraffin sections 5 μm thick were stained with Mayer hemalum and eosin.

TABLE 1. Strains of mice inoculated with *Cryptosporidium* sp. at 1 to 4 days of age

Mice	Inoculum no.	Infection[a]
Experimental		
Random Porton	1	+
Schneider Swiss White	1	+
Inbred Porton	1	+
BALB/c	2	+
CBA Nude	3	+
Hairless (HR/HR-ADR)	3	+
C57 Black	3	+
CBA	3	+
Control	None	−

[a] +, *Cryptosporidium* sp. detected by histology and fecal examination; −, no *Cryptosporidium* sp. detected.

RESULTS

Susceptibility of mouse strains. All eight strains of mice inoculated between 1 and 4 days of age were susceptible to *Cryptosporidium* sp. infection (Table 1), although none of the mice had clinical illness at any time. Fecal cryptosporidia were detected from 3 to 7 days p.i.

When examined by light microscopy, villi in the terminal ilea and the luminal surfaces of the ceca were extensively covered with cryptosporidia, but the duodena were only sparsely infected. Significant pathological changes were not observed at any intestinal site in mice infected with *Cryptosporidium* sp.

Histological and fecal examination did not detect infection in uninoculated age-matched mice from the same isolators as inoculated animals.

Age-related susceptibility. Mice inoculated at 4 days of age became heavily infected in the ilea, but only mild infections were established in 21-day-old mice (Table 3). Histological examination correlated well with fecal detection of the organism, but infected areas of gut could be detected histologically after fecal shedding of the organism had ceased (Table 3).

Hairless (HR/HR-ADR) and Swiss White mice inoculated at 21 to 42 days of age did not

TABLE 2. Freezing and freeze-drying methods used to store *Cryptosporidium* sp.

No.	Cryopreservative Description[a]	Storage conditions
1.	5% PVP	Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C
2.	5% Glycerol	Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C
3.	10% DMSO	Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C
4.	10% DMSO + 20% BSA	Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C
5.	SPGA + DMSO to 8% (vol/vol)	Rapid cooling in liquid nitrogen, then slow thaw at 15–20°C
6.	20% NRS + 10% glycerol (equilibrated at 15 to 20°C for 20 h)	Slow cooling to −70°C, then slow thaw at 15–20°C
7.	10% DMSO	−20°C for 3 days, slow thaw
8.	20% Glycerol	−20°C for 3 days, slow thaw
9.	PBS	−20°C for 14 days, slow thaw
10.	PBS	−70°C for 14 days, slow thaw
11.	10% Glycerol	Freeze-drying for 2 days at 4°C

[a] Abbreviations: PVP, Polyvinylpyrrolidone (molecular weight, 44,000); DMSO, dimethyl sulfoxide; SPGA, per liter 74.6 g of sucrose, 0.52 g of KH_2PO_4, 1.25 g of K_2HPO_4, 0.91 g of L-glutaric acid, 10 g of BSA; NRS, normal rabbit serum.

become infected (total of 28 mice examined), and infection was detected in only 1 CBA Nude mouse 9 days p.i. (total of 14 mice examined).

Immunosuppressed mice. The cyclophosphamide treatment was effective in reducing the louping-ill antibody response from a value of 160 to 320 in normal mice to one of 10 to 40 in immunosuppressed mice. However, no mice in any group became infected with *Cryptosporidium* sp.

Effect of multiple passage on pathogenicity. The calf *Cryptosporidium* sp. isolate remained infective for mice after 16 mouse passages over a 3-month period. Two gnotobiotic lambs 2 days old that were given inoculum 3 developed diarrhea and pathological lesions typical of those described for cryptosporidiosis in experimental lambs (22).

Storage of *Cryptosporidium* sp. Freezing destroyed the activity of *Cryptosporidium* sp. irrespective of the cryopreservation method used (Table 2).

There was a progressive loss of infectivity in all media at 4°C (Fig. 2). No infectivity was detectable after 2 months of storage in distilled water; the most stable preparation was in 2.5% potassium dichromate, in which infectivity lasted 4 to 6 months. Complete loss of infectivity occurred at 15 to 20°C within 2 weeks and at 37°C within 5 days.

DISCUSSION

These experiments demonstrate that *Cryptosporidium* sp. isolates can subclinically infect eight different strains of SPF mice 1 to 4 days old, but the same strains at 21 days of age or older have a lower susceptibility to infection. This age-related susceptibility could explain why some workers using *Cryptosporidium* sp. isolated from guinea pigs (26) and cats (9) failed

TABLE 3. Age-related susceptibility to infection by *Cryptosporidium* sp.

Age at inoculation (days)	Infection at day p.i.[a]: 3	5	6	7	8	10	11	12	13	14	16
4	−	++	+++	+++	+++	+++	+++	++	+	+	+
21	−	−	NT	+	+	−	+	−	−	−	NT

[a] Two mice were killed on each day from each age group. Symbols: −, No infection as detected by fecal examination or histology; NT, not tested; +, mild infection as detected by histology; ++, mild infection as detected by histology plus fecal shedding of *Cryptosporidium* sp.; +++, moderate to heavy infection as detected by histology plus fecal shedding of *Cryptosporidium* sp.

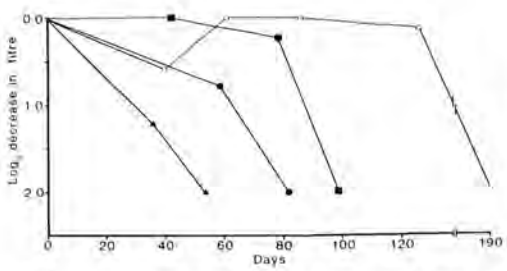

FIG. 2. Infectivity of a calf *Cryptosporidium* sp. isolate for mice after storage for different periods of time at 4°C in laboratory media. Titers were measured in 50% mouse infective doses in distilled water (▲), 5% BSA (●), PBS (■), and 2.5% potassium dichromate (△).

to infect weanling mice or 7-week-old ICR mice, respectively. This effect has been recognized before (8, 19), but recently adult mice have been infected with *Cryptosporidium* sp. of human and calf origin (15), and hence, the infectivity of the inoculum is important.

When considering the extent of infection present in the ilea and ceca of 1- to 4-day-old mice, it is surprising that no pathological changes occur and that only subclinical infections can be established. This situation is in contrast to the severe clinical disease and pathological changes observed in young lambs infected with the organism (22).

Cryptosporidium sp. infections have been observed in immunosuppressed humans (5, 10, 28), but no enhanced susceptibility could be shown in mice treated with cyclophosphamide, a procedure which impaired the antibody response to louping-ill virus vaccine. The reduction in antibody response to virus served as an indicator of general antibody depression, but this apparently failed to increase the susceptibility of weanling mice to *Cryptosporidium* sp. infections. Presumably, factors other than antibody-mediated responses are involved in resistance to these infections. With one exception, all CBA Nude mice inoculated at 28 days of age were apparently not susceptible to *Cryptosporidium* sp. infection.

The calf *Cryptosporidium* sp. isolate used was passaged 12 times in mice and still remained pathogenic for gnotobiotic lambs. It is not known whether this capability applies to all *Cryptosporidium* sp. isolates, but its presence could be advantageous in maintaining and storing field isolates and in providing a biological filtration system to remove enteric viruses (e.g., rotavirus and coronavirus) or pathogenic bacteria (e.g., enterotoxigenic *Escherichia coli*). Hence, an inoculum devoid of any other known enteropathogens could be established.

Continuous biological passage is time consuming and costly, and there would be many advantages in being able to store strains provided their pathogenicity could be maintained. The various cryopreservatives used failed to preserve the organism at low temperatures, although two of the methods used (Table 2, numbers 5 and 6) have been successful in long-term storage of rickettsiae (3) and *Toxoplasma gondii* (6), respectively. Storage of the *Cryptosporidium* sp. isolate at 15 to 20 or at 37°C in PBS resulted in inactivation within 2 weeks, but at 4°C the organism remained viable for at least 3 to 4 months. Heine and Boch (8) also reported that calf feces containing *Cryptosporidium* sp. stored at 4°C remain viable for 3 to 6 months. These findings may have epidemiological implications in that the organisms may survive in feces for a considerable period in a temperate climate.

The use of the mouse titration method to quantitate a *Cryptosporidium* sp. inoculum was preferred to the flotation method described by Iseki (9), which in our experience yields only a small percentage of the cryptosporidia in feces. Moreover, it is impossible to assess the percentage of viability in the sample since it has been shown that flotation can affect viability (8). With the mouse titration method, an overall viability, measured as the 50% mouse infective dose, is obtained for each inoculum, so that comparisons between inocula can be made.

The sensitivity of the mouse titration method was improved by employing histological examination of the gut as an index of infection rather than relying on Giemsa staining of fecal smears. The histological findings in selected titrations correlated with smear results (data not shown), but in general the percentage of infected animals detected on histological examination was higher.

In conclusion, laboratory mice are a very convenient animal model which may facilitate the study of many aspects of *Cryptosporidium* sp. infections which are not clearly understood, such as the immunology and the life cycle of the organism.

ACKNOWLEDGMENTS

The authors thank the staff of the Clinical Studies Department for supplying the SPF mice and for maintaining the gnotobiotic lambs, and Barbara Shaw for the louping-ill virus vaccine work. D.S. is in receipt of an Agricultural Research Council studentship grant.

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J. Comp. Path. 1984, Vol. 94.

EXPERIMENTAL CRYPTOSPORIDIOSIS IN GERMFREE LAMBS

By

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INTRODUCTION

Cryptosporidium is an enteric pathogenic coccidium for man (Bird and Smith, 1980; Tzipori, Angus, Gray and Campbell, 1980b; Weinstein, Edelstein, Madara, Falchuk, McManus and Trier, 1981; Anderson, Donndelinger, Wilkins and Smith, 1982; Reese, Current, Ernst and Bailey, 1982), calves (Pohlenz, Moon, Cheville and Bemrick, 1978b; Snodgrass, Angus, Gray, Keir and Clerihew, 1980a; Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980c), lambs (Angus, Appleyard, Menzies, Campbell and Sherwood, 1982a) and many other species. Enteritis in both natural and experimental infections has been described, but because of the presence of other intestinal micro-organisms conclusive proof of the causal relationship between the parasite and the lesions has not been obtained (Pohlenz, Bemrick, Moon and Cheville, 1978a; Tzipori, Angus, Gray and Campbell, 1981; Moon and Bemrick, 1981). Recently, enterocolitis due to *Cryptosporidium* in gnotobiotic piglets has been described (Tzipori, Smith, Makin and Halpin, 1982).

The objective of this study was to attempt to remove contaminating microorganisms from cryptosporidia-containing faeces and to investigate the disease produced in germfree lambs by the resulting "clean" suspension of cryptosporidia.

MATERIALS AND METHODS

Preparation of Inoculum

The *Cryptosporidium* isolate used was obtained from the faeces of a 2-week-old calf with diarrhoea. After storage for 4 months at 4 °C 1 ml of faeces was given orally to a gnotobiotic lamb which was treated orally thrice daily with 5 mg gentamicin and 50 mg ampicillin. Cryptosporidia were detected in the faeces of this lamb 4 days after dosing, and the bacterial contamination was reduced to a *Bacillus* sp. The *Cryptosporidium* was then passaged sequentially through 2 further gnotobiotic lambs, each of which received oral treatment thrice daily with gentamicin, ampicillin, 200 mg streptomycin, 25 mg oxytetracycline and 10 000 units nystatin. On the third lamb passage, antibiotic treatment was discontinued after 6 days, and 24 h later a large volume of faeces containing cryptosporidia was collected from this lamb. These faeces were homogenized with 4 volumes of phosphate buffered saline, and stored at 4 °C for 7 to 10 weeks before being used as inoculum for the subsequent experiments.

The inoculum was checked for bacterial growth under both aerobic and anaerobic conditions. It was inoculated onto foetal bovine kidney and foetal ovine kidney cell cultures to detect cytopathic viruses (Snodgrass, Herring, Reid, Scott and Gray, 1980) and

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was also examined by electron microscopy to detect enteric viruses (Snodgrass, Smith, Gray and Herring, 1976). No contaminating micro-organisms were detected.

The inoculum was titrated in half-log dilutions in 3-day-old inbred Porton mice, 0·1 ml volumes being inoculated intraoesophageally into each mouse. Each dilution was given to all the mice in one litter. The titre was stable throughout the experimental period at $10^{3\cdot3}$ 50 per cent mouse infective doses per ml. The number of cryptosporidia in the inoculum estimated from counting 5μl drops dried on glass slides was approximately $10^{5\cdot3}$ per ml.

Animals

The experimental design is detailed in Table 1. Eight 2-day-old gnotobiotic lambs housed in pairs in plastic isolators were dosed orally with either 0·5 or 1·0 ml of the *Cryptosporidium* inoculum. Three lambs (nos 6 to 8) were simultaneously dosed with 1·0 ml of a mixture of equal amounts of 6 h cultures of *Proteus* sp., *Streptococcus* sp. and 2 non-enterotoxigenic *Escherichia coli*, isolated from a healthy 1-week-old calf. Two inoculated lambs (nos 4 and 5) were treated twice daily with 10 mg gentamicin to ensure longer term bacteria-free status. One gnotobiotic control lamb was also treated with gentamicin. Tissues from uninoculated control gnotobiotic lambs from previous experiments served as a guide to normal intestinal structure (Snodgrass, Ferguson, Allan, Angus and Mitchell, 1979; Gray, Angus, and Snodgrass, 1980).

Accidental contamination with bacteria occurred in only one lamb. This was lamb no. 3 whose intestinal contents obtained at necropsy 48 h post-inoculation (p.i.) contained very small numbers of a *Bacillus* sp. which grew only after 2 days incubation in nutrient broth.

Observations

Daily faeces samples from all lambs were smeared on glass slides, air dried, fixed in methanol, stained by Giemsa's method, and examined for cryptosporidia.

Bacterial isolation techniques were also used on these samples. The lambs were observed daily, particular attention being paid to the volume and appearance of the faeces. Voluntary milk intake was recorded.

Abomasal and intestinal samples were collected under terminal pentobarbitone anaesthesia. Intestinal sites sampled were: descending duodenum (1); jejunum, approximately 50 cm distal to the duodenojejunal flexure (2); midgut (3); distal ileum, from areas both with and without Peyer's patches (4, 5); caecum (6); and spiral colon (7). Samples for histopathological, immunofluorescent (IF) and transmission electron microscopic (TEM) examination were processed as described previously (Gray *et al.*, 1980; Snodgrass, Angus and Gray, 1977). The serum used for IF staining was obtained from a gnotobiotic lamb 19 days after infection with the third gnotobiotic lamb passage of calf *Cryptosporidium*.

For scanning electron microscopy (SEM), 6 mm^2 pieces of intestine were fixed as for TEM, dehydrated in a graded acetone series, and dried by the critical point method from liquid carbon dioxide. Tissues were sputter-coated with gold and examined in a Cambridge Stereoscan S180 SEM.

To determine what stages were present in faeces, samples were suspended in 1 per cent glutaraldehyde and centrifuged at 15 000 *g* for 2 min; the pellet was then processed for TEM examination.

RESULTS

Clinical

Lambs nos 1, 2 and 3 killed up to 48 h p.i. remained bright, showed normal appetite, and passed firm brown faeces. All other infected lambs developed clinical signs, although these varied considerably in extent between individuals.

The presence of a contaminating "normal" gut flora in lambs 6 to 8 did not exacerbate their clinical signs. The most consistent sign was reduced appetite, usually commencing on the third day p.i. (Fig. 1). The duration varied from lamb 3 whose appetite returned to normal within 2 days, to lamb 8 which still showed much reduced appetite 11 days after onset. During this period, lambs were apparently hungry and keen to feed until they had drunk a small volume of milk, at which point they lost appetite.

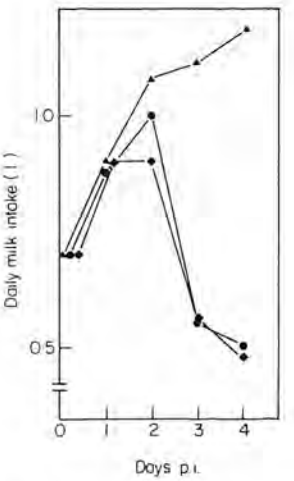

Fig. 1. Voluntary milk intake of infected lambs (mean values) for 4 days following infection with *Cryptosporidium*. The control data are derived from 9 uninfected age-matched gnotobiotic lambs from previous experiments. ▲——▲ Control lambs; ●——● infected lambs (bacteria free); ◆——◆ infected lambs (contaminated).

Profuse watery diarrhoea developed 3 days p.i. in lambs 4 and 6, but faeces of lambs 5, 7 and 8 remained formed, although looser than normal. Lambs 6 and 8 became dull, but other lambs remained alert. Lamb 6 died of *E. coli* septicaemia 3 days p.i.

Faecal excretion of cryptosporidium. Cryptosporidia were observed in faecal smears from infected lambs from the third or fourth day p.i. until the lambs were killed. Cryptosporidia were not detected in the control lamb (no. 9), nor have we ever seen them in gnotobiotic lambs.

Development of early cryptosporidial infection (up to 48 h p.i.). By light microscopy a few trophozoites were detected only in the midgut of lamb 1 killed 12 h p.i. In lamb 2, killed 24 h p.i., endogenous stages of *Cryptosporidium* were detected sparsely in midgut and ileum, with trophozoites predominating. At 48 h p.i. in lamb 3 a few organisms were seen in the duodenum, and numbers increased distally in the small intestine, although the large intestine was not infected at this stage. Schizont stages as well as trophozoites were now recognizable.

By TEM, endogenous stages of *Cryptosporidium* observed were similar morphologically to those described in cats (Iseki, 1979) and man (Bird and Smith,

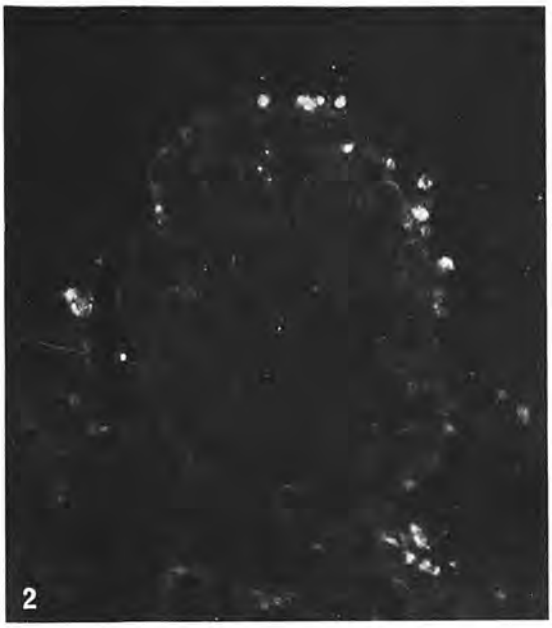

Fig. 2. Immunofluorescent-stained villus from ileum of lamb 3 killed 48 h p.i. Individual parasites are attached to the apical margin of enterocytes. × 300.

TABLE 1

DOSAGE AND TIME OF NECROPSY OF LAMBS AFTER BEING GIVEN A SUSPENSION OF CRYTOSPORIDIUM

Lamb no.	*Suspension volume (ml)*	*Gentamicin treatment*	*Time killed (h p.i.)*
1	1·0	–	12
2	1·0	–	24
3	1·0	–	48
4	0·5	+	144
5	0·5	+	192
6	1·0 + 1·0 ml bacteria†	–	72
7	1·0 + 1·0 ml bacteria	–	144
8	1·0 + 1·0 ml bacteria	–	288
9	Control lamb	+	168*

* Age (h).
– No gentamicin treatment; + gentamicin treatment.
† The component bacteria are detailed in the test.

1980). The sequential development of these stages up to 48 h and later is summarized in Table 2. Cryptosporidia were first detected in the lamb killed 24 hours p.i., mainly as trophozoites. A 30-fold increase in the number of organisms took place between 24 and 48 h p.i. Although all endogenous stages were apparent at this period, the proportion of trophozoites remained about 90 per cent.

No immunofluorescence was observed in lambs 1 and 2 killed 12 and 24 h p.i.,

respectively. Specific discrete staining of *Cryptosporidium* in the epithelial brush border (Fig. 2) was first observed in midgut and ileum of lamb 3 killed 48 h p.i.

Later cryptosporidial infection (from 72 h p.i.). In lambs killed from 72 to 288 h p.i. cryptosporidia were found at all small and large intestinal sites examined, although they were consistently most numerous in midgut and ileum (Fig. 3). All life cycle stages other than microgametocytes were observed (Table 2). In some instances organisms were present in crypts as well as on surface mucosa.

Fig. 3. Ileum heavily infected with cryptosporidia. SEM × 1800.

TABLE 2

NUMBERS AND STAGES OF CRYPTOSPORIDIUM OBSERVED BY TEM IN ILEUM OF DOSED LAMBS

Hours (p.i.)	*No. observed in 50 fields*	*Percentage of observed organisms appearing as:*				
		Trophozoite	*Schizont*	*Macrogamete*	*Microgametocyte*	*Zygote*
12	0	0	0	0	0	0
24	51	91	9	0	0	0
48	1521	88	8	1	1	1
72	1922	35	8	51	0	6
144	1734	50	4	38	0	8

Later stages of the organism were often found to have parallel vertical folds extending externally from the attachment zone (Fig. 4). These folds were seen both by SEM and TEM. Fusion of microvilli to the outer surface of organisms was not seen although they were often in contact.

Structure of faecal stages. The only intact stages seen in faecal pellets were

Fig. 4. Vertical corrugations in the outer wall of the parasite. SEM × 25 000.

oocysts containing 4 naked sporozoites and a residual body, enclosed by a laminated membrane. In some planes of section, the outer lamina formed ridges or spiky projections. Comparison between these exogenous stages (Fig. 5) and zygotes containing developing sporozoites (Fig. 6) suggested a possible mechanism of oocyst release by rupture of the outer, host-derived enclosing membrane.

Morphological changes: light microscopy and SEM. The distribution and severity of changes associated with *Cryptosporidium* infections in the lambs is shown in Table 3. No morphological changes were seen in the intestines of lambs nos 1, 2 and 3, killed up to and including 48 h p.i. or in the control lamb (no. 9) treated with gentamicin. Extensive mucosal changes similar to those described in more conventional lambs (Angus, Gray and Tzipori, 1982b) were associated with the presence of numerous cryptosporidial organisms in all other infected lambs. Severe villus stunting and fusion (Figs 7 and 8) with replacement of columnar and mucus-secreting cells by cuboidal cells, were invariably found in the ileum, with copious infiltrates of mononuclear cells. In lambs nos 4 and 7, these changes were also seen in the midgut. Infections were also widespread in the caecum and spiral colon, where there was dilatation or atrophy of the crypts, with infiltrates of mononuclear cells.

Lesions in lambs nos 6 to 8, which had been infected with "normal" calf gut bacteria in addition to *Cryptosporidium*, did not differ either qualitatively or in extent from those in lambs 4 and 5, which remained bacteria-free. No adherent bacteria were seen in any site.

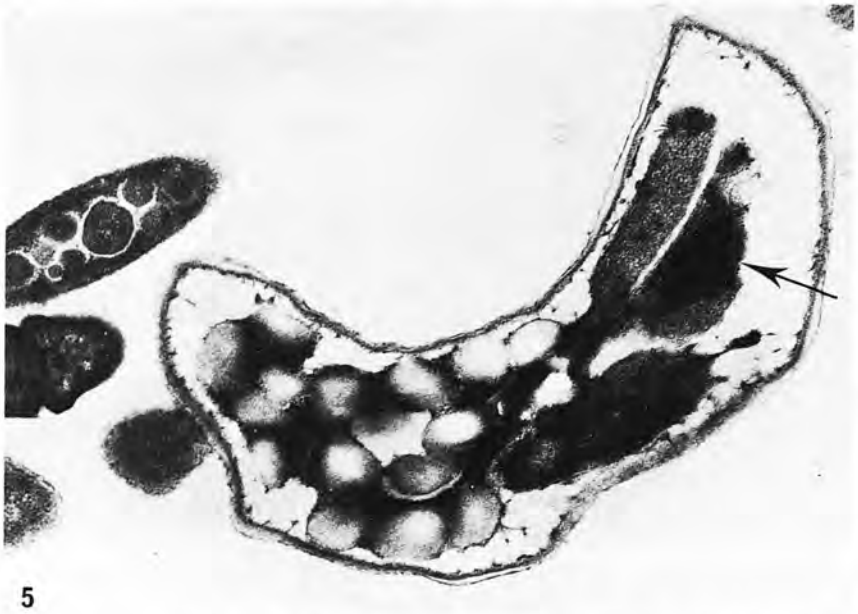

Fig. 5. Typical oocyst in faecal pellet. Note the sporozoites and residual body enclosed by a laminated membrane. Arrow indicates sporozoite nucleus. TEM × 30 000.

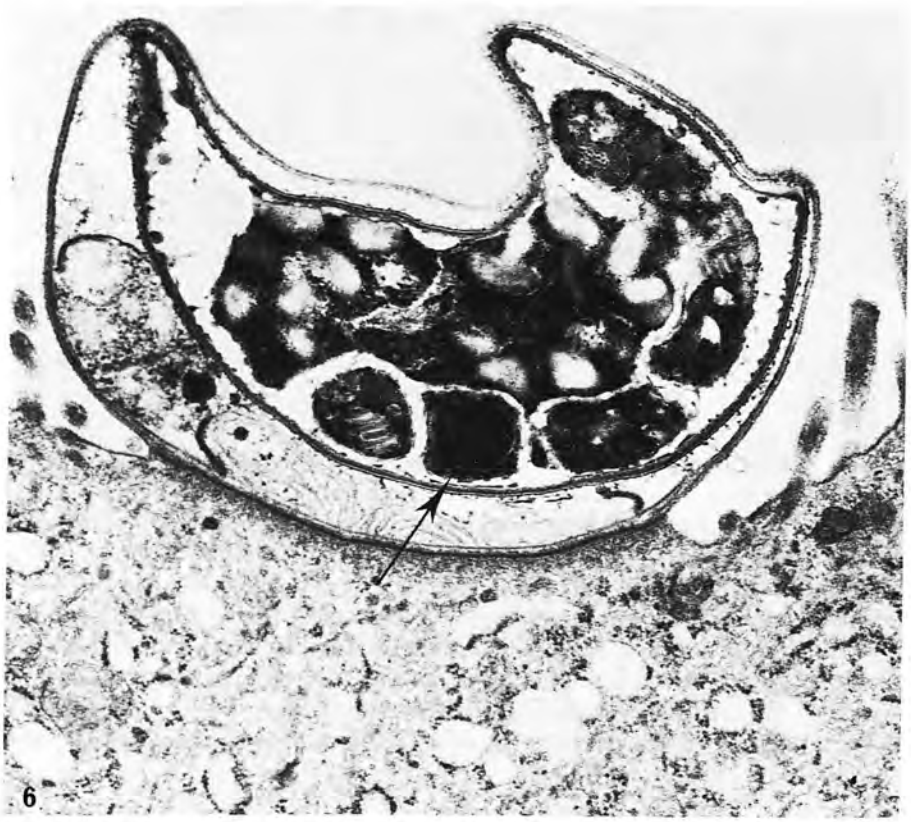

Fig. 6. Zygote in faeces containing developing sporozoites and residual body. Rupture of the outer membrane causes shedding into the gut lumen of the structures enclosed by the inner laminated membrane. Arrow shows sporozoite nucleus. TEM × 30 000.

TABLE 3

RELATIONSHIP BETWEEN INFECTION WITH CRYPTOSPORIDIUM AND DEGREE OF MUCOSAL DAMAGE IN THE SMALL INTESTINE (SITES 1–5), CAECUM (SITE 6) AND SPIRAL COLON (SITE 7) OF LAMBS

Lamb number (h p.i.)	*Degree of infection** / *Degree of mucosal damage†* at intestinal sites sampled						
	1	2	3	4	5	6	7
1 (12)	–	–	+	–	–	–	–
	–	–	–	–	–	–	–
2 (24)	–	–	+	+	+	–	–
	–	–	–	–	–	–	–
3 (48)	+	+ +	+ + +	+ + +	–	–	–
	–	–	–	–	–	–	–
4 (144)	+	+	+ +	+ + +	+ + +	+ + +	+ + +
	–	+	+ +	+ + +	+ + +	+ +	+ +
5 (192)	+	+	+	+	+ + +	+ + +	+ + +
	–	–	–	+	+ + +	+	+
6 (72)	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+
	+	+	+	+ + +	+ + +	–	–
7 (144)	+	+	+ + +	+ + +	+ + +	+ + +	+ +
	–	–	+ + +	+ + +	+ + +	+ +	+ +
8 (288)	+	+	+ +	+ + +	+ + +	+	+ +
	+	–	–	–	–	+	+
9 (control)	–	–	–	–	–	–	–
	–	–	–	–	–	–	–

* + + + = Heavy infection; + + = moderate infection; + = light infection.
– = No cryptosporidia seen.
† + + + = Severe villus atrophy and fusion with cellular infiltrates; + + = moderate villus atrophy with cellular infiltrates (SI sites) or diltatation of crypts with moderate cellular infiltrates (LI sites); + = minor morphological changes with light cellular infiltrates.
– = No morphological changes.

DISCUSSION

In the absence of any detectable virus or bacteria, *Cryptosporidium* from a calf consistently produced enteric lesions and anorexia in germfree lambs, and caused watery diarrhoea in some, thus confirming the large amount of circumstantial evidence that *Cryptosporidium* can act as a primary enteric pathogen. The enteric disease produced was not exacerbated by the presence in 3 lambs of bacterial gut flora from a healthy calf, although one lamb developed septicaemia.

The lesions due to cryptosporidiosis in the germfree lambs are similar to those described in conventional animals (Angus *et al.*, 1982b). However, the sequential development of lesions from the incubation period through clinical disease is described for the first time. Initial multiplication during the incubation period took place primarily in midgut and ileum. At the onset of disease at about the

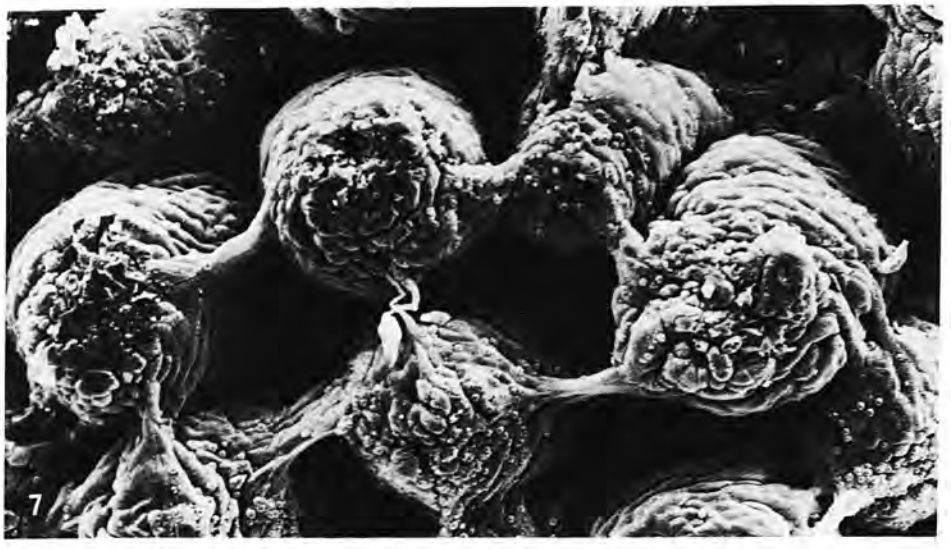

Fig. 7. Stunting and crossbridging of villi in the midgut of an infected lamb, 196 h p.i. SEM × 500.

Fig. 8. Midgut of infected lamb 5 killed 192 h p.i., showing villus fusion, partial atrophy and cellular infiltration. HE × 150.

third day after infection, lesions were widespread in the small and large intestine, although of greatest severity in the ileum. Although lambs were observed for up to 12 days after infection, normal appetite did not return in some cases nor did the gut lesions heal in that period.

The development of lesions occurred later than the large increase in

cryptosporidial numbers. At 48 h p.i. cryptosporidia were present in large numbers in morphologically normal gut, while at 72 h p.i. widespread damage was associated with similar cryptosporidial numbers. It may be that lesions are produced particularly during gametogony rather than by trophozoites which predominate earlier. This would be in agreement with observations in other coccidial species (Todd and Ernst, 1977).

Our studies revealed a life cycle development as described for feline cryptosporidiosis (Iseki, 1979). We did not observe a second generation schizont containing 4 merozoites as suggested previously by other investigators (Vetterling, Jervis, Merrill and Sprinz, 1971; Pohlenz *et al.*, 1978a). The function of the external folds was not ascertained but may be an aid to attachment or stability. Some other workers have not observed oocysts in faeces (Bird and Smith, 1980; Vetterling *et al.*, 1971), but in our study typical oocysts were observed. Ultrastructural examination of faecal pellets confirmed that most organisms in faeces are oocysts (Reese *et al.*, 1982; Pohlenz *et al.*, 1978a), although it must be expected that other stages will be excreted on exfoliated epithelial cells.

The extent to which *Cryptosporidium* produces disease in man and animals remains to be determined. The possibility that cryptosporidia from different species are in fact identical, and that only one species exists (Tzipori, Angus, Campbell and Gray, 1980a) adds considerable epidemiological interest. Even a substantial infection of the intestine does not always lead to clinical disease, as we have found in mice and some experimental calves. Indeed, lambs may be unusual in being particularly susceptible to cryptosporidiosis (Angus *et al.*, 1982b). However, there seems little doubt that cryptosporidial infection is becoming recognized as a cause of enteritis in man and his domesticated animals, and the demonstration of pathogenicity in germfree lambs supports this contention.

SUMMARY

Contaminating bacteria were removed from an isolate of calf *Cryptosporidium* by 3 sequential passages of the parasite in gnotobiotic lambs, together with antibiotic treatment of the lambs. This preparation, which contained no detectable bacteria or viruses, was given by mouth to 8 2-day-old gnotobiotic lambs, 3 of which were dosed at the same time with bacterial flora from a healthy calf. Lambs were killed at intervals from 12 to 288 h post-inoculation and the sequential development of the parasite, of enteric lesions, and of clinical illness was observed. Lesions were characterized by severe villus stunting and fusion. Clinically the most consistent sign was anorexia, with some lambs developing also a severe watery diarrhoea. Lesions and clinical signs were similar in lambs with and without intestinal bacteria. This demonstration of the enteropathogenicity of *Cryptosporidium* in germfree lambs suggests that it is a pathogen of significance.

ACKNOWLEDGMENTS

We thank A. Ross and MRC Unit of Clinical and Population Cytogenetics at the Western General Hospital, Edinburgh for providing generous access to their scanning electron microscope.

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[*Received for publication, January 21st,* 1983]

CRYPTOSPORIDIOSIS

INTEREST in diagnosing particular infectious diseases waxes and wanes, and a fashionable agent at the moment is the coccidian parasite *Cryptosporidium*, discovered in 1907.[1] The stimuli for this interest are probably twofold—the recognition of cryptosporidiosis as a disease of veterinary importance, particularly in calves;[2-4] and the severe symptoms produced by cryptosporidiosis in immunocompromised patients, particularly those with acquired immunodeficiency syndrome (AIDS).[5]

The parasite's life-cycle is direct, through faecal-oral transmission, and is typical of Eimeriidae with asexual and sexual stages of reproduction. Characteristically the endogenous stages (2–6 μm in diameter) are enclosed in a parasitophorous vacuole in the microvillous border of enterocytes in small and large intestine.[6] However, other epithelial surfaces have on occasion been infected, particularly bile and pancreatic ducts, gallbladder, and respiratory tract.[5,7,8]

Cryptosporidiosis in man manifests as two clinically distinct conditions—either a self-limiting gastroenteritis in normal patients, or a chronic life-threatening diarrhoea in immunologically compromised individuals. The cases of infection in immunologically normal patients that have so far been reported in detail number thirty-two.[9-17] Ages ranged

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from 2 months to 35 years, and all but two cases were symptomatic, with a self-limiting influenza-like gastrointestinal illness. Diarrhoea was the outstanding symptom, but most patients also had fever, abdominal pain, and nausea, and constipation was occasionally reported. Duration of diarrhoea ranged from 3 to 10 days, and all patients recovered. In addition to these cases there is information from three surveys of patients with gastrointestinal symptoms in the UK, Australia, and Finland.[18-20] Of nearly 3000 stool samples examined, slightly over 2% contained cryptosporidial oocysts. Infection was more common in children than in adults.

There are published records of forty-nine immunocompromised patients with cryptosporidiosis.[5,17,21-31] Most of these patients had AIDS, but others had immunoglobulin deficiencies or were under immunosuppressive therapy. All patients had severe protracted watery diarrhoea (up to 12 litres/day), often with fever, substantial weight loss (up to 50% of initial weight), abdominal pain, and lymphadenopathy. Diarrhoea commonly continued intermittently or continuously for many months. Infection in such patients was usually fatal, although most had other conditions associated with their immunodeficiencies. One patient recovered after discontinuation of immunosuppressive therapy.[21]

No therapeutic agent has been found effective in treating natural cases of cryptosporidiosis in man or animals, although a wide range of antiprotozoals and antibacterials has been tried.[27] In experimental infections in calves and mice many drugs have been used both prophylactically and therapeutically, but no satisfactory treatment has so far been found.[32-34] However, in immunologically normal humans and animals, disease is self-limiting; patients make a spontaneous clinical recovery and parasite excretion in stool becomes undetectable. In immunocompromised patients, on the other hand, the outlook is grave. Fluid and electrolyte therapy and parenteral nutrition have been successful in sustaining patients for several months, but in these cases cryptosporidiosis has persisted until death.

Although there are clear differences in biological behaviour between different strains,[4] many strains from animals and man can be shown to infect and cause disease in species other than the original host.[12,17,35] Cryptosporidiosis thus has potential to be a zoonosis, and many of the sporadic cases in man have been in individuals in contact with animals—notably calves, which were in some cases known to be infected.[11,12,17] However, these animal-associated cases may have assumed an undue importance owing to the greater interest of the veterinarian in diagnosing the condition. Certainly in most of the immunocompromised patients there was no history of animal contact,[5] and direct human-to-human transmission can occur.[15,16]

Diagnosis depends on demonstration of the parasite, either of endogenous stages in situ on intestinal mucosa obtained by biopsy or at necropsy, or more commonly of oocysts in stool. Oocysts can be identified by flotation and examination by phase-contrast microscopy,[36] or by staining with Giemsa, Ziehl-Neelsen, or various other methods and examination by light microscopy.[37-39] With practice the oocysts can be readily recognised as 4 μm diameter spherical structures, typically with an unstained peripheral halo, and up to four sporozoite nuclei and the residual body may be visible within the oocyst. Despite the interest in cryptosporidiosis as a disease, studies on *Cryptosporidium* as a parasite are in their infancy. At the Animal Diseases Research Association's Moredun Institute in Edinburgh, techniques have been developed for oocyst purification and enumeration, for excystation of sporozoites, and for infection in cell culture and embryonated eggs. These should allow much-needed progress to be made in biological and antigenic strain comparison, in immunological studies, and in in-vitro screening of potential therapeutic agents.

Cryptosporidium is thus not an exotic parasite of marginal clinical interest, but one that will take its place as a recognised human pathogen. What that place is, remains to be defined: the dramatic symptoms of cryptosporidiosis in AIDS cases, while instrumental in bringing the condition to the forefront, may turn out to be of no more overall importance than the widespread but much milder cases of gastroenteritis in normal children or adults. Cryptosporidiosis as a possible pathogen in the much greater problem of diarrhoea in the developing countries also remains to be assessed.

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Shiga-like toxin production from *Escherichia coli* associated with calf diarrhoea

D. Sherwood, D. R. Snodgrass, A. D. O'Brien

Veterinary Record (1985) **116**, 217

ANIMAL and human enteropathogenic *Escherichia coli* have been extensively studied and characterised. Some strains invade the intestinal epithelium (enteroinvasive *E coli*) (Formal and others 1976), while others are non-invasive and produce gut adhesive antigens and enterotoxins (enterotoxigenic *E coli* [ETEC]) (Moon 1974). However, other serotypes of human *E coli* that are consistently associated with diarrhoea are neither enteroinvasive nor enterotoxigenic (Rowe and Gross 1983) but elaborate a Vero cell cytotoxin (Konowalchuck and others 1977). This toxin has been shown to be identical to the shiga toxin produced by *Shigella dysenteriae* (O'Brien and others 1982, O'Brien and LaVeck 1983, O'Brien and others 1983a, b).

Such strains have been isolated from the faeces of humans and calves with diarrhoea (Scotland and others 1979, Wilson and Bettleheim 1980), and from pigs with oedema disease and diarrhoea (Smith and others 1983). In addition, studies on a rabbit *E coli* strain (Cantey and Inman 1981) and various human and pig *E coli* strains (Moon and others 1984) suggest that *E coli* attaching to and effacing intestinal microvilli may also produce a toxin similar to shiga toxin.

Isolates of *E coli* from diarrhoeic calves were screened for K99 antigen, heat stable and heat labile enterotoxins and for cytotoxic activity on Vero cells (Sherwood and others 1983). Vero cytotoxicity was detected in one ETEC isolate. The isolation of Vero cytotoxic but non-enterotoxigenic *E coli* from diarrhoeic calves in the same study is reported here. Thirteen such strains were isolated from nine of 306 (3 per cent) diarrhoeic calves. The O serogroups of these isolates are shown in Table 1. Two calves were concurrently infected with *Cryptosporidium* species and one with rotavirus and coronavirus.

TABLE 1: Cytotoxic activity of *E coli* producing shiga-like toxin

Isolate O serogroup	Cytotoxicity for HeLa cells of Supernatant fluid (CD50/ml)	Bacterial lysate (CD50/mg protein)
O4	1×10^4	$2{\cdot}38 \times 10^4$
O8	$5{\cdot}12 \times 10^3$	$1{\cdot}5 \times 10^3$
O19	$1{\cdot}28 \times 10^3$	$4{\cdot}69 \times 10^2$
O26	1×10^6	$4{\cdot}24 \times 10^7$
O26	1×10^6	$6{\cdot}02 \times 10^5$
O111	1×10^7	$3{\cdot}45 \times 10^7$
O111	1×10^7	$4{\cdot}22 \times 10^6$
O111	$5{\cdot}12 \times 10^3$	$2{\cdot}67 \times 10^3$
O149	1×10^4	$5{\cdot}68 \times 10^3$
O168	$2{\cdot}56 \times 10^3$	$8{\cdot}26 \times 10^3$
O serogroup undetermined	$2{\cdot}56 \times 10^3$	$6{\cdot}1 \times 10^3$
	1×10^6	$1{\cdot}28 \times 10^6$
	1×10^6	$1{\cdot}18 \times 10^6$

CD50 Cytotoxic dose of supernatant fluid or bacterial lysate required to kill 50 per cent of HeLa cells in a microtitre assay. The relationship to shiga toxin was shown by neutralisation of 10 CD50 of toxin by antiserum to shiga toxin but not by normal rabbit serum

The cytotoxic activities of bacterial broth culture supernatant fluids and of bacterial cell lysates were determined on HeLa cells. Rabbit antiserum to purified shiga toxin (O'Brien and others 1982) was used to neutralise this cytotoxicity. All 13 isolates produced a cytotoxin which in all cases was neutralised by antiserum to shiga toxin but not by normal rabbit serum (Table 1).

This study indicates that a small proportion of diarrhoeic calves is infected with *E coli* that produce a shiga-like toxin. Serogroups O26 and O111 isolated from five calves in this study have also been associated with shiga-like toxin production in *E coli* recovered from diarrhoeic humans (Scotland and others 1979, Wilson and Bettleheim 1980, Karmali and others 1983). Hence it is possible that calves may harbour *E coli* capable of producing enteric infections in man.

Acknowledgements.— The authors thank Dr B. Rowe (Central Public Health Laboratory, Colindale, London) for the O serogrouping results.

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THE VETERINARY RECORD, NOVEMBER 21, 1981

Papers and Articles

Inheritance of *Escherichia coli* K88 adhesion in pigs: Identification of nonadhesive phenotypes in a commercial herd

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Veterinary Record (1981) **109**, 461-463

A method for preparing fragments of brush border from the small intestinal epithelial cells of pigs was modified so that specimens as small as 3 mm × 3 mm could be used. This modification also allowed more rapid preparation and the brush borders thus prepared adhered specifically to K88+ but not K88− *Escherichia coli*. At slaughter 12·2 per cent of 459 bacon weight pigs from a commercial herd were of nonadherent phenotype. Litters containing only nonadherent pigs were identified. Parents of these litters and siblings intended for breeding stock replacements could be identified as probably also being of nonadherent phenotype and this was confirmed by examining biopsy samples obtained by enterotomy from the siblings.

THE K88 pilus antigen of *Escherichia coli* is a virulence determinant, its adhesive properties enabling bacterial attachment to piglet intestinal epithelium (Jones and Rutter 1972). K88 strains of *E coli* are common among swine throughout the world, although at least two other pilus antigens, K99 and 987P, also confer the ability to adhere (Moon and others 1980).

K88+ *E coli* adhere to brush borders prepared from small intestinal epithelial cells from most but not all pigs (Sellwood and others 1975). These two phenotypes are the products of two alleles at a single locus which are inherited in a simple Mendelian manner, with adherence (S) being dominant over nonadherence (s) (Gibbons and others 1977, Sellwood and Kearns 1979). Susceptibility to diarrhoea caused by both natural and experimental infection with K88+ *E coli* is limited to piglets of adherent phenotype (Rutter and others 1975, Sellwood 1979).

The objective of this work was to develop techniques that could be used to determine the phenotype of pigs in commercial piggeries, to enable selection of nonadherent pigs from within the existing breeding stock.

Materials and methods

Piggery

These studies were conducted on a closed herd of 3200 sows of large white, landrace and mixed breed, housed in four units. The work was largely confined to a unit of 800 sows containing the nuclear large white and landrace breeding stock.

Neonatal diarrhoea was continuously present on the farm although prevalence varied. Recent litter records showed that piglets in 1508 of 3609 litters (44 per cent) were treated for neonatal diarrhoea. *E coli* isolated from scouring piglets on this farm possessed the K88 antigen (S. Tzipori, personal communication).

Brush border preparation

The technique of Sellwood and others (1975) was used to prepare brush borders from small intestine. In addition, two methods requiring smaller pieces of intestine were evaluated. Initially, brush border preparations were made for comparison from the duodenum, jejunum, midgut and distal ileum of 11 pigs. Subsequent preparations were made routinely from the duodenum.

Initially, segments of small intestine approximately 3 mm × 3 mm were incubated with K88+ or K88− *E coli* for 30 minutes at 37°C, washed twice in phosphate buffered saline and frozen in a dry ice-isopentane sludge. Cryostat sections were stained for immunofluorescent examination with a rabbit anti-K88 antiserum and fluorescein-conjugated anti-rabbit immunoglobulin.

Subsequently, a micromodification of the technique used by Sellwood and others (1975) was developed and used routinely. All steps were performed at 4°C except where stated otherwise. Segments of intestine, approximately 10 mm^2 were excised and kept in 0·15M sodium chloride until processed within eight hours of collection. The saline solution was replaced by a solution containing 0·096M sodium chloride, 0·008M potassium hydrogen phosphate, 0·0056M sodium hydrogen phosphate, 0·0015M potassium chloride and 0·01M ethylene diamine tetracetate (EDTA), pH 6·8, for 15 minutes at room temperature. The mucosal surface of the specimen was then scraped with a scalpel blade into 5 ml of a solution similar to the above except that it contained 0·3M sucrose in place of EDTA. This cell suspension was homogenised in a Teflon tipped tissue grinder by moving the pestle up and down six times while it rotated at about 1000 rpm (Sellwood and others 1975). The homogenate was centrifuged at 1200 *g* for 10 minutes and the pellet resuspended in 5 ml 0·005M EDTA, pH 7·4 (adjusted with 0·5M sodium carbonate). Homogenisation was repeated and the suspension centrifuged at 300 *g* for four minutes. The pellet was then washed in Krebs-Henseleit buffer, pH 7·4, containing 0·12M sodium chloride, 0·014M potassium chloride, 0·025M sodium bicarbonate and 0·001M potassium hydrogen phosphate and resuspended in that buffer to give approximately 10^7 fragments/ml.

E coli

Strains were grown overnight in tryptone soya broth (Oxoid) at 37°C, washed twice in Krebs-Henseleit buffer and

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resuspended to approximately 5×10^8 organisms/ml. K12 strains with and without K88 plasmid (Nagy and others 1977) and a K88+ strain WG isolated from diarrhoeic pigs (Tzipori and others 1980) were used.

Adhesion assay

Twenty-five microlitres of brush border suspension was added to each of three wells of a microtitre plate followed by 25 µl of *E coli* suspension. The plates were sealed and incubated for 30 minutes at room temperature on a rotary agglutinator oscillating at 25 rpm. A drop of the brush border preparation, and of each of the three brush border-bacteria suspensions, was dried on a microscopic slide, fixed in methanol and stained with Giemsa.

Sampling at slaughter

Bacon weight pigs from the unit were slaughtered on one day each week. On two successive weeks, samples of duodenum were obtained from all pigs slaughtered. The litter identification number from each pig was noted.

Biopsy procedure

Small intestinal biopsies were obtained from 12 experimental pigs and seven on-farm pigs weighing from 20 to 100 kg. Pigs were fasted for 24 hours, premedicated with azaperone (Stresnil; Ethnor) at 4 mg/kg intramuscularly and anaesthesia was induced and maintained with halothane (Fluothane; ICI). A laparotomy incision was made, from 2 cm posterior to the umbilicus, in the midline in females and lateral to the prepuce in males. A small section of adjacent small intestine was exteriorised and an approximate 10 mm^2 segment of gut was removed from the antimesenteric border. The intestine was closed with Cushing sutures using 000 Vicryl (Ethicon). The abdomen was closed routinely and antibiotics were administered parenterally for three days. Normal feeding was recommenced the day after operation.

Results

Brush border preparation and adhesion

After incubation of intestinal segments with K88+ *E coli*, adhesion could be demonstrated in immunofluorescent sections. However, the technique proved time consuming and relatively difficult to interpret.

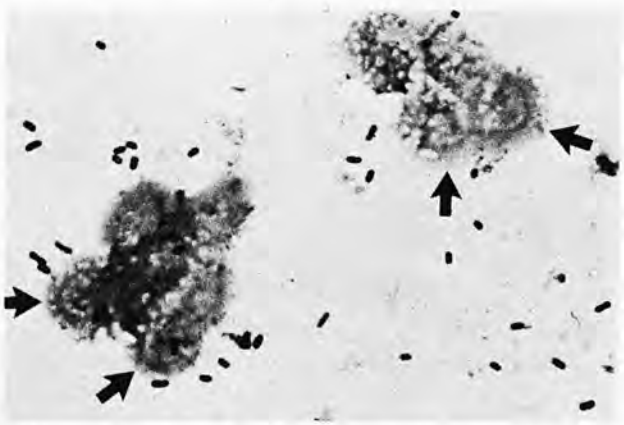

FIG 1: Brush borders from pig of adhesive phenotype after incubation with K88− *E coli*. The characteristic convex margins of the fragments are arrowed. × 630

The micro technique was effective at producing cellular fragments, most of which could be identified as brush borders after staining with Giemsa (Fig 1). The borders had a characteristic morphology, typically with a convex margin on which the microvilli could be observed as striations. The technique of Sellwood and others (1975), on which our procedure was based, also produced good brush border preparations but the micro technique proved much quicker to perform.

After incubation with K88− *E coli*, no brush borders prepared from any pig attached to the bacteria (Fig 1). Brush borders from adherent type pigs when incubated with K88+ *E coli* strains were covered with closely adherent bacteria (Fig 2). This reaction was prevented by prior incubation of *E coli* with antiserum to K88, but not to K99 or 987P. Brush borders from certain pigs did not react with K88+ *E coli* and these pigs were taken as the nonadhesive phenotype.

Segments from all parts of small intestine examined proved to be of equal value in preparing brush borders for the assay.

Prevalence of nonadhesive phenotypes in the herd

Brush border preparations were made from duodenal samples from 459 pigs at slaughter, of which 56 (12·2 per cent) were shown by the microtechnique to be nonadherent. The litter number of each pig could be identified from ear marks, and the result from all tests were allocated to individual litters.

It was found retrospectively that between two and six pigs had been sampled from each of 145 litters. Four of these litters (2·7 per cent) contained only nonadherent phenotype pigs, 36 litters (25 per cent) contained both adherent and nonadherent phenotypes, while in 105 litters (72 per cent) all pigs were of adherent phenotype.

As nonadherence is the expression of homozygous recessive (ss) genotype, a litter of nonadherent phenotype is produced by ss parents. Thus the identification of nonadherent litters at slaughter enabled parents and siblings on the farm to be phenotyped as nonadherent also. By this method, two boars, three sows and six siblings were identified as of probably nonadherent phenotype. In addition, eight other boars sired a high proportion of segregating litters, indicating a genotype of either Ss or ss.

Biopsy results

All pigs recovered uneventfully and continued to thrive. At necropsy of two of the pigs several months later only minor adhesions with no stenosis were present.

Samples taken from the 12 experimental pigs by enterotomy proved suitable for brush border preparation.

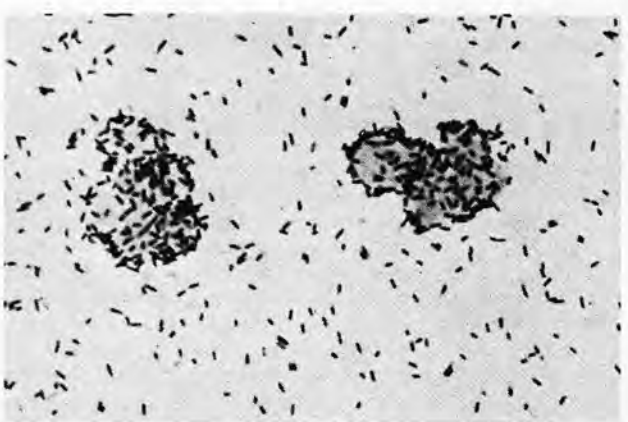

FIG 2: Brush borders from pig of adhesive phenotype after incubation with K88+ *E coli*. × 420

Nine pigs were of adherent phenotype and three of nonadherent phenotype.

Seven siblings from nonadherent litters identified from the slaughterhouse survey had been selected for breeding stock on the basis of conformation and performance testing. Biopsy results from these confirmed that five gilts and one boar were of nonadherent phenotype, while one gilt was of adherent phenotype.

Discussion

The micro technique for preparing brush borders proved to be reliable, specific and relatively quick to perform. Its use provided a means of examining a large number of samples collected from the slaughterhouse, and samples obtained by biopsy.

The survey of slaughtered pigs showed only 12·2 per cent (1:8·2) of pigs from this farm unit were of nonadhesive phenotype. From random matings only 1·5 per cent (1:67) of litters could be expected to contain only nonadhesive phenotype pigs. The actual figure of 2·7 per cent nonadhesive litters was likely to be too high, as some segregated litters may have been missed due to the relatively low number of pigs sampled per litter. This was in fact shown to be the case by the detection on biopsy of one adhesive phenotype pig, reducing the wholly nonadhesive litters to 2·1 per cent of the total.

Biopsy by enterotomy proved to be safe and effective and confirmed the results from the survey. Had more pigs per litter been sampled at slaughter, biopsy might have been unnecessary. However, our concern was to establish the validity of both methods of determining phenotype, either directly through biopsy or indirectly through sampling of progeny or siblings at slaughter. Both these methods proved to be practicable.

The basic objective was to provide the pig farmer who is reluctant to buy boars of nonadherent phenotype, which may not meet the standards of conformation or performance of his herd, with the alternative of identifying nonadherent animals from within his existing breeding stock. The methods outlined in this paper show that this alternative approach can be successfully applied.

Acknowledgements. — The authors wish to thank the management of Mayfair Farms, Huntly for all cooperation. Technical assistance was provided by J. Arnott, J. Billington and K. Wilson. The work was supported in part by the Australian Pig Industry Research Committee.

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Abstracts

Leptospires in English pigs

SERA from 597 pigs killed at three abattoirs in the south of England were examined by the microscopic agglutination test at a dilution of 1:100 against six pools of live antigens representing a total of 16 serogroups of *Leptospira*. Kidneys of the same pigs were examined bacteriologically. Ninety-four (15·7 per cent) of the sera had leptospiral titres and leptospires were isolated from five kidneys; four of the isolates were *icterohaemorrhagica* and one was the *hebdominis* group. The commonest titres in sera were to *bratislava*.

HATHAWAY, S. C., LITTLE, T. W. A. & STEVENS, A. E. (1981) *Research in Veterinary Science* **31,** 169

Epidemiology of *Uncinaria stenocephala*

FROM May to the following February, faeces from dogs naturally infested with *Uncinaria stenocephala* were, at monthly intervals, placed on grass in a clean paddock in south-east England. From one week after deposition of the faeces, grass samples were collected at intervals of two weeks during the summer and monthly during the winter. In summer, larval development and translation occurred in as little as one week but took up to five months during winter. Overwintered larvae persisted until May or June, when they were replaced by a new generation of larvae.

WALKER, M. J. & JACOBS, D. E. (1981) *Research in Veterinary Science* **31,** 264

Antibiotic resistance in bacteria isolated from meat

TWENTY strains each of *Escherichia coli* and *Salmonella typhimurium* isolated from pig carcases or meat products were tested for antibiotic sensitivity and their ability to transfer resistance factor. All strains of both groups were 100 per cent resistant to penicillin-G and vancomycin, but very sensitive to trimethoprim and nadilixic acid. The resistance transfer value between *E coli* and *E coli* K12 was 30 per cent, between salmonella and *E coli* K12 was 25 per cent and between *E coli* and *Salmonella typhimurium* was 25 per cent. The results underline strongly the need to use antibiotics and similar drugs with the greatest caution in treatment.

BIRU, G., SEEGER, H. & GEMMER, H. (1981) *Deutsche tierärztliche Wochenschrift* **88,** 181

Milk as substrate for diagnosing EBL

ENZYME-linked-immunosorbent assay (ELISA) using milk was compared with immunodiffusion on bovine serum for the diagnosis of enzootic bovine leukosis. ELISA detected 85·1 per cent of infected animals, immunodiffusion only 76·2 per cent, the difference being statistically significant. The increased sensitivity depends purely on the detection of smaller amounts of antibody. The possibility of use of milk for enzootic bovine leukosis diagnosis under practice conditions must await study of possible variables such as the stage of lactation, the duration of infection and the usefulness of bulk milk samples.

MANZ, D. (1981) *Deutsche tierärztliche Wochenschrift* **88,** 169

Research in Veterinary Science 1987, 43, 72–77

Enteric campylobacter infection in gnotobiotic calves and lambs

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Gnotobiotic calves and lambs were infected orally with *Campylobacter jejuni*, *C coli* or *C hyointestinalis* to assess pathogenicity. All animals were successfully colonised and excreted mucoid faeces but showed no other clinical signs. Campylobacters colonised the large intestine better than the small intestine, in which bacterial numbers decreased with time after infection. Campylobacters were found occasionally in the lumen of crypts in close proximity to epithelial cells and included in a mucus-like material. Lesions were mostly in the large intestine in calves whereas in lambs they were present in the ileum. In animals inoculated with *C jejuni* or *C coli* scattered crypt abscesses, focal inflammatory infiltrates in the lamina propria and goblet cell discharge were found. In lambs inoculated with *C hyointestinalis* only minor changes were found in the small intestine. Serum antibody response was either absent or present at a low level only from the 19th day after infection.

THAT campylobacters might be a cause of calf scour was first suggested by Jones and Little (1931). However, exposure of young calves to *Campylobacter jejuni* or *C coli* inconsistently produced either diarrhoea (Firehammer and Myers 1981) or ileitis and mucoid faeces (Al-Mashat and Taylor 1980); similar changes were produced in lambs with *C jejuni* or *C coli* (Firehammer and Myers 1981) and in milk-fed or ruminant calves with other campylobacters (Al-Mashat and Taylor 1981, 1983). In all cases the disease was slight and without mortality.

Many of these studies were carried out using young conventionally reared ruminants, sometimes infected with other enteropathogens and of uncertain immune status. For these reasons the present experiments to investigate the pathogenicity of campylobacters for neonatal calves and lambs and to seek for differences in pathogenicity between bacterial strains were undertaken with gnotobiotes.

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Materials and methods

Bacterial strains

The strains used to infect animals and other experimental details are listed in Table 1. Identification was based on the methods of Karmali and Skirrow (1984) and Gebhart et al (1983), and serotyping of *C jejuni* and *C coli* was carried out by the passive haemagglutination technique of Penner and Hennessy (1980).

Experimental animals and diet

Calves were caesarian-derived, colostrum-deprived and lambs were hysterectomy-derived, colostrum-deprived (Hart et al 1971). All animals were maintained under gnotobiotic conditions (Coates 1968) in plastic isolators and were bottle-fed on evaporated cows' milk two or three times daily according to a schedule based on age.

Design of experiments

Three gnotobiotic calves (1 to 3) (Table 1) kept in separate isolators were infected orally with *C jejuni*. Calf 1 was inoculated with a strain from an outbreak of calf neonatal diarrhoea, calves 2 and 3 were both infected with the same strain reisolated from gnotobiotic calf 1 after storage (−80°C) and a single subculture. Lambs were infected with one or other of two strains of *C jejuni* biotype 1, *C coli* or *C hyointesinalis* and were maintained in six isolators (Table 1).

Clinical examinations

Animals were inspected daily, milk intake was noted and compared with a standard schedule. Faecal consistency and mucus content were recorded daily and every second day samples were taken for bacterial enumeration.

Necropsy

Animals were removed from the isolator and blood

was taken from the jugular vein. Terminal anaesthesia was then induced with intravenous sodium pentobarbitone, the abdominal cavity opened and portions of tissues taken aseptically from the following sites: pyloric area of abomasum, duodenum, jejunum and mid gut, lower ileum without (site 1) and with (site 2) Peyer's patches, colon, caecum and rectum, mesenteric lymph nodes (MLN), liver, spleen and lung. Gastrointestinal segments were ligated before removal, transferred to individual containers on ice and processed as soon as possible; the MLN and the organs were frozen at −80°C and examined later.

Adjacent portions of the gastrointestinal tissues were fixed in 10 per cent formol phosphate buffered saline and processed in standard fashion for light microscopy. All tissues were stained with Mayer's haematoxylin and eosin; in some selected sections Young's or Kerr's modification of the Warthin-Starry technique and the periodic acid Schiff were carried out. Tissues in chilled 0·1 M cacodylate buffer at 4°C, were diced into 2 mm^3 blocks, fixed in 2·5 per cent gluteraldehyde in 0·1 M cacodylate buffer (pH 7·3) and processed for transmission electron microscopy (TEM). Pieces of bowel of 2 to 3 cm in length were opened, immediately frozen in liquid nitrogen, and subsequently processed (Pope et al 1979) for scanning electron microscopy (SEM).

Bacteriological techniques

Weighed samples of faeces or paramucosal material from the gastrointestinal sites were suspended in phosphate buffered saline (PBS 0·1 M, pH 7·2) and the samples homogenised for 30 to 40 seconds at 13,000 *g* in an MSE homogeniser. Enumeration of bacteria was carried out on appropriate dilutions by the method of Miles et al (1938). Portions of other tissues were placed in PBS (10 per cent w/v), homogenised and a loopful plated. Cultures for the detection of *C hyointestinalis* were incubated at 37°C for 42 hours in a 3 per cent oxygen, 10 per cent carbon dioxide, hydrogen rich atmosphere (Lawson and Rowland 1974) and those for *C jejuni* or *C coli* at 43°C for 20 hours in a similar atmosphere but with 6 per cent oxygen. Campylobacters and contaminant bacteria were counted on 5 per cent horse blood agar (BABA, Oxoid CM331 or CM271) with added 0·2 per cent (w/v) agar (Oxoid, L11) and on two selective media which were (i) Preston medium (Bolton and Robertson 1982) modified by the addition of 0·05 per cent of each of iron-bisulphate pyruvate (FBP) compound (Skirrow and Benjamin 1980) and nystatin (100 iu ml^{-1}) to replace cyclohexamide for counting of *C jejuni*; (ii) Skirrow's medium (Butzler and Skirrow 1979) with 0·2 per cent agar for enumeration of *C coli* and *C hyointestinalis*.

To detect contaminants rectal swabs were placed in nutrient broth (Oxoid CM67), sodium thioglycollate (0·11 per cent w/v) nutrient broth and Sabouraud liquid medium (Oxoid CM147). All media were incubated at 37°C for up to 10 days. If turbidity in nutrient broth or the thioglycollate broth was observed, subcultures were made on CBA and incubated aerobically or anaerobically, respectively.

Inocula

All strains were recovered from diarrhoeic calves involved in outbreaks (Snodgrass et al 1986). After purification they were frozen at −80°C in vials containing tryptose phosphate broth (Oxoid CM282),

TABLE 1: Identification of gnotobiotic calves and lambs, distribution of animals in isolators, age of animal when inoculated and killed and serological identification of strains

Animal number	Isolator	Inocula: *Campylobacter* species	Strain number	Serotype	Dose given (log_{10})	Age of animals in days at: Inoculation	Necropsy
Calf 1	A	*C jejuni* biotype 1	D523-2	4,13,16,34,	9·3	1	8
Calf 2	B			43,50,26	9·3(M), 10·0(N)*	1	15
Calf 3	C				9·5(M), 8·9(N)	1	22
Lamb 1	D	*C jejuni* biotype 1	D523-2	4,13,16,34	8·4	1	5
Lamb 2	E			43,50,26	8·8	1	20
Lamb 3	E				8·8	1	31
Lamb 4	F	*C jejuni* biotype 1	B2454-2	4,13,16,34	8·6	1	4
Lamb 5	F			43,50,26	8·6	1	8
Lamb 6	G	*C coli*	D531-32	20	8·6	1	4
Lamb 7	H				9·0	2	17
Lamb 8	H				9·0	2	28
Lamb 9	I	*C hyointestinalis*	B293-19	—	9·1	1	7
Lamb 10	I				9·1	1	14
Lamb 11	I				9·1	1	26

* (M) Mucoid and (N) non-spreading colonies

5 per cent (v/v) horse serum and 17 per cent (v/v) tyndallised glycerol. Before infection, vials were thawed, campylobacters cultured on CBA, sub-cultured in a modified brucella broth medium (Difco 0495) incorporating 0·3 per cent yeast extract powder (Oxoid L21), 0·15 per cent (w/v) agar (Oxoid L33) and 0·05 per cent of each FBP compound in the appropriate atmosphere and temperature for the strain, as previously indicated.

Calves and lambs were infected orally with 10 ml or 1 ml of cultures, respectively, the numbers of campylobacters given being enumerated by viable counts on BABA.

Serological procedures

Humoral antibodies to whole-cell antigens of campylobacter were detected with a technique modified from Lawson and Rowland (1974). The *C hyointestinalis* strain was harvested and diluted with 0·3 per cent formol PBS (pH 7·2, 0·01 M) but for the *C jejuni* (B2454-2) and *C coli* strains the formol concentration was increased to 0·5 per cent to reduce autoagglutination. Stable agglutination could not be prepared for *C jejuni* D523 even using this method. Equal volumes (0·5 ml) of standard diluted antigen were added to doubling dilutions of sera in PBS in the wells of WHO plates and the test incubated at 37°C overnight.

Statistics

Log counts of campylobacter in faeces and gastro-intestinal segments were compared by Student's *t* test or Duncan's multiple range test (Harter 1960).

Results

Infection of calves with C jejuni

The calves showed no depression or illness, diarrhoea was not detected and food consumption was normal. Faeces were soft, never fluid and intermittently contained mucus from the first or second day after inoculation until the end of the experiment; in calf 3 streaks of fresh blood were seen on days 3 to 4 after inoculation.

All three calves were colonised rapidly by the organism, which was detected in the faeces from day 1 after inoculation; thereafter excretion was continuous at levels between 10^8 and 10^{10} organisms g^{-1}. Two colony types, both biochemically *C jejuni* biotype 1 and the same serotype, were detected in the faeces of calves 2 and 3; one was the typical spreading mucoid colony (M) and the other a small, non-spreading raised colony (N). SEM showed the M colony contained flagellated and the N colony largely unflagellated cells. Both types of colony were

TABLE 2: Mean counts ($log_{10}g^{-1}$) of campylobacters in representative gastrointestinal segments in infected calves and lambs

Animal		Organism	Day after infection*	Abomasum	Duodenum	Jejunum	Mid-ileum	Lower ileum (1)	Lower ileum (2)	Colon	Caecum	Rectum
Calf	1	*C jejuni* D523-2	7	5·6	5·8	5·2	5·9	6·6	7·3	9·3	9·5	—
	3		21	5·2	4·0	3·8	4·3	1·9	1·9	8·5	7·9†	7·9
Lamb	1	*C jejuni* D523-2	4	4·4	5·6	3·7	4·7	5·4	7·0	10·1	9·8	—
	3		30	3·7	2·3	2·0	4·1	3·9	5·0	9·3	9·1†	7·2
	4	*C jejuni* B2464-2	3	5·8	5·8	5·8	6·9	7·0	7·9	8·9	8·8	—
	5		7	6·5	4·8	2·7	2·7	4·9	5·9	7·8	10·0	—
	6	*C coli*	3	3·9	5·7	3·6	2·8	4·6	5·6	8·5	8·2	—
	8		28	2·7	2·7	2·7	4·2	4·4	5·1	9·2	8·9	9·0
	10	*C hyointestinalis*	13	3·8	4·5	3·8	4·1	3·9	5·0	8·5	8·7	5·2
	11		25	2·8	2·7	2·7	2·7	3·2	5·0	9·0	8·6	8·2

— Not tested
* Day of necropsy
† Reduction in bacterial counts from intestinal sites between calves 1 and 3 and between lambs 1 and 3 significant ($P<0·01$)

FIG 1: Caecum of calf 1 showing a necrotic crypt containing neutrophils and local inflammatory infiltrate extending through the muscularis mucosae. H&E × 340

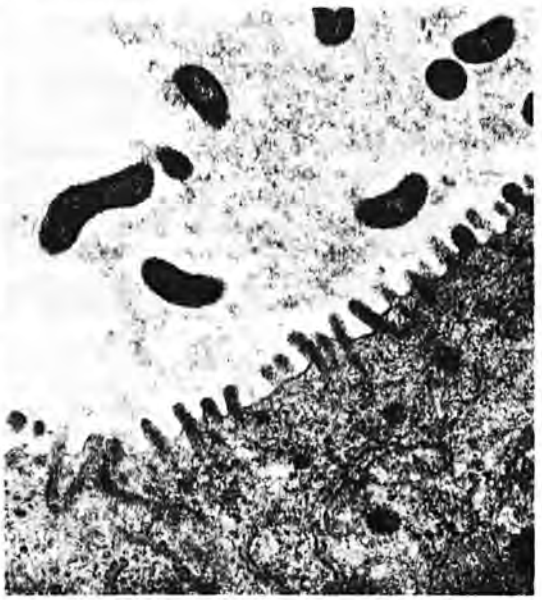

FIG 2: Caecal crypt of calf 1 showing campylobacters in the lumen in close proximity to the surface epithelium and lying in an amorphous mucus-like material. TEM × 6000

recovered throughout but neither consistently predominated.

Calf 1 remained uncontaminated during the experimental period, calf 2 became contaminated by *Bacillus* species (day 3 after inoculation) and a coagulase-negative staphylococcus (day 12); calf 3 was contaminated by a coagulase-negative staphylococcus (day 3) and *Clostridium perfringens* (day 13).

At necropsy all gastrointestinal sites yielded *C jejuni*, the greatest numbers being present in the large intestine. There was a significant reduction in the numbers of campylobacters in the intestinal tract with time ($P<0{\cdot}01$), this effect being most noticeable in the lower levels of the ileum (Table 2). N type predominated over M type colonies at most sites except the rectum. The organism was isolated from all the extraintestinal tissues in calf 2 but only from the MLN of calf 3.

There were no macroscopic abnormalities in the alimentary tract of any of the calves. However, the contents of the large bowel were mucoid or gelatinous in all three calves. Microscopic lesions were mainly localised to the large intestine and consisted of scattered, dilated or damaged crypts containing abundant neutrophils (crypt abscesses), with associated inflammatory infiltrates in the surrounding lamina propria (Fig 1). In calf 2 the colon showed some oedema and focally the epithelium was cuboidal and devoid of goblet cells. PAS staining demonstrated the presence of adherent mucus overlying the mucosal surface. Proctitis was also present in this calf. Silver stains demonstrated campylobacters in the large intestine but only occasionally in the small intestine. The organisms were generally present singly or in small groups, adjacent to but not attached to the mucosal surface and embedded in an amorphous mucus-like material. Only in the large intestine and rectum of calves 1 and 2 were organisms occasionally numerous. TEM and SEM confirmed the location and distribution of bacteria seen by light microscopy; the organisms were located in an amorphous granular material and were separated from the microvillous surface (Fig 2).

Agglutinating antibodies were detected only in calf 3 at low titre (1/40).

Infection of lambs with C jejuni

The lambs were not noticeably clinically affected and their appetites remained normal. All five animals produced soft, semi-formed mucoid faeces from day 2 after inoculation onwards.

All animals became colonised by *C jejuni* and

faecal excretion was present from day 2 after inoculation onwards; in those animals where counts were made numbers ranged from 10^9 to 10^{10} campylobacters g^{-1} for strain D523. At necropsy the pattern of recovery was similar to that seen in calves with the largest numbers of bacteria being present in the large intestine and a tendency for numbers to fall with time after infection ($P<0{\cdot}01$), particularly in the small intestine. Both strains of *C jejuni* colonised to a similar pattern. Excretion of M and N colony types varied but at necropsy N colonies were more numerous at all sites except two in the two lambs in which this point was studied. *C jejuni* was isolated irregularly from the visceral tissues of lambs 1, 4 and 2 but only from the MLN of 3 and 5.

No macroscopic abnormalities were found in any lamb. In all animals the contents of the large intestine and faeces had a mucoid consistency. Some incipient crypt abscesses were found in the ileum of lambs 1 and 5 and the caecum of lamb 5. Histological, SEM and TEM studies did not reveal any other relevant abnormalities. Campylobacters and other bacteria were occasionally encountered in the lumen of glands in the large intestine of all lambs; the campylobacters were very often embedded in an amorphous mucus-like material but none was seen attached to or in epithelial cells.

Infection of lambs with C coli *or* C hyointestinalis

Clinical and microbiological results of infection with *C coli* or *C hyointestinalis* were similar to those of *C jejuni*, the salient point being the lack of clinical effect. Persistent excretion was noted, with, at necropsy, widespread intestinal infection numerically highest in the large bowel and a decrease in small intestinal infection with time. Differences were apparent in the excretion of the species of campylobacters in those animals in which this was measured. The mean numbers per gram differed significantly and were $10^{9{\cdot}8}$ for *C jejuni*, $10^{9{\cdot}2}$ for *C coli* and $10^{7{\cdot}5}$ for *C hyointestinalis*. It was clear, however, that although this pattern was present in all the animals in which excretion was quantified, highly significant differences in excretion were present between individual animals infected with *C hyointestinalis* ($P<0{\cdot}001$).

Minor histological changes were found only in the caecum and rectum of lamb 7 infected with *C coli*. These consisted of incipient 'crypt abscesses' and focal subepithelial neutrophil aggregates. Only a few campylobacters were found similarly located to *C jejuni* within mucus associated with the luminal surface of the large intestine.

Similar pathological changes were absent from lambs infected with *C hyointestinalis* and changes were only encountered in the jejunum and the ileum (site 2) of lambs 10 and 11: principally the presence of shortened or dome-shaped villi in site 2. In silver-stained sections campylobacters could not be detected in association with the epithelium in these animals.

Agglutinating antibody was not detected using the homologous species as antigen in sera from lambs killed on or before day 14. Lambs infected for longer periods had antibody titres between 1/40 and 1/80.

Single or dual contamination with bacteria and, on one occasion, a yeast often occurred in lambs with initial infection being generally detected on days 2 to 3 after inoculation. These infections involved *Bacillus* species, *Streptococcus* species, coagulase-negative staphylococcus and *Escherichia coli*. There was no evidence that the presence of the contaminating bacteria influenced colonisation by campylobacters.

Discussion

Animals successfully colonised by *C jejuni*, *C coli* and *C hyointestinalis* but showed only a mild, subclinical disease characterised by alterations in faecal consistency and minor pathological changes. Lambs infected with *C hyointestinalis* excreted fewer campylobacters suggesting that this *Campylobacter* species is less well adapted to the ovine intestinal tract. No difference was found between the two *C jejuni* strains tested and calf passage did not seem to modify substantially the pathogenicity of one of them. Colonisation was more marked in the large than in the small intestine, as with infection in gnotobiotic piglets (Kashiwazaki et al 1971), dogs (Prescott et al 1981) and newborn rodents (Field et al 1981) and unlike conventional adult mice in which the number of *C jejuni* was greater in the small than in the large intestine (Blaser et al 1983). Comparable, quantitative information is not on record for conventional or adult calves or sheep. It was a constant finding that the numbers of campylobacters decreased with time in the small but not in the large intestine.

Newell et al (1983) found that flagellate/motile *C jejuni* colonised the intestinal tract of very young mice better than non-motile variants. In the experiments with calves and lambs, N colonies were more numerous than M colonies in most gastrointestinal segments, suggesting that motile variants had no particular advantage in the colonisation of young, gnotobiotic ruminants.

In all animals the faeces and large intestinal contents were mucoid and on one occasion blood was noted, features previously reported in conventional calves and lambs infected with campylobacters. The presence of mucus in calf 1 and lamb 6 monoinfected with *C jejuni* and *C coli*, respectively, confirms that these bacteria are responsible for its release. Cholera toxin has been shown specifically to cause mucus release from goblet cells (Elliot et al 1970). A cholera-

like toxin has been isolated from *C jejuni* (Ruiz-Palacios et al 1983), and a similar toxin may have been present in the campylobacter strains used in these experiments. Campylobacters were never found intracellularly but they were occasionally noted in the lumen of crypts in close proximity to epithelial cells and consistently included in a mucus-like material, confirming previous observations that camplyobacters are mucosa-associated microorganisms (Lee et al 1983). SEM fixation of tissues was carried out to preserve mucus but only fragments of it were obtained instead of the layers described in rodents (Field et al 1981), which may indicate a different texture of mucin in ruminants.

In calves the lesions were mainly in the large intestine whereas in lambs the changes were mainly localised in the ileum; this difference could relate to the species, or perhaps to the presence of contaminant microorganisms in the lambs which, unlike the calves, harboured other agents either before inoculation or in the early stages of infection. The minor lesions in the *C jejuni* or *C coli* infections were similar to the changes in human campylobacter colitis or proctitis (Price et al 1979). *C hyointestinalis* infections in lambs were almost without lesions. *C hyointestinalis* has been linked with the intracellular campylobacter associated disease of pigs, proliferative enteropathy (Chang et al 1983). A pathologically similar disease has been reported in lambs (Hoorens et al 1977) but the absence of intracytoplasmic campylobacters or significant changes in lambs infected with *C hyointestinalis* does not support any association between this organism and the disease in lambs. Altogether, the lesions were milder than those described in conventional calves infected with *C jejuni* or other campylobacters (Al-Mashat and Taylor 1980, 1981, 1983) and in no case was the abomasum or jejunum involved.

Antibody response was poor but was detected from day 19 after inoculation onwards. This result is in agreement with reports of campylobacter infections in gnotobiotic piglets (Kashiwazaki et al 1971) but differs from those obtained with experimental infections in conventional calves (Al-Mashat and Taylor 1980, 1981) in which higher titres were detected.

Acknowledgements

The Moredun Institute procured and maintained the gnotobiotes. Dr L. Roberts from the Veterinary Investigation Centre, Bucksburn, Aberdeen serotyped the campylobacter strains.

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Received April 1, 1986
Accepted September 1, 1986

APPENDIX PAPER A

Journal of Virological Methods, 00 (1988) 000–000
Elsevier

JVM 00694

Evaluation of an immunogold electron microscopy technique for detecting bovine coronavirus

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(Accepted 15 December 1987)

Summary

A solid phase colloidal gold immunoelectron microscopy (IGEM) technique for detecting bovine coronavirus (BCV) was developed and shown to be specific. This test was compared with three other diagnostic tests using fifteen faecal samples. Bovine coronavirus was detected in 2 samples by direct electron microscopy (DEM), in 3 samples by immunosorbent electron microscopy, in 5 samples by haemadsorption-elution-haemagglutination and in 6 samples by IGEM. Ninety four faecal samples were tested by DEM and IGEM. Of 26 samples found to contain BCV by IGEM only 14 were positive by DEM. The IGEM technique is simple, efficient and less susceptible than others to non-specific reactions.

Bovine coronavirus; Immunogold; Electron microscopy, Diagnosis

Introduction

Neonatal calf diarrhoea is a syndrome of complex aetiology in which several infectious agents interact with environmental influences to determine the health of the young calf. Bovine coronavirus (BCV) is an endemic virus known to be involved in calf diarrhoea (Bridger et al., 1978; Mebus et al., 1973; Patel et al., 1982; Reynolds et al., 1986; Saif et al., 1986; Snodgrass et al., 1986; Stair et al., 1972), and has been isolated also from the respiratory tract (McNulty et al., 1984; Saif et al., 1986). In two recent studies BCV was detected in 4% of 302 diarrhoeic calves in Scotland and the north of England (Snodgrass et al., 1986) and 14% of 490 diarrhoeic calves in the south of England and Wales (Reynolds et al., 1986).

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Several methods have been used to detect BCV in faeces. With direct electron microscopy (DEM) (Reynolds et al., 1984) it is often difficult to differentiate pleomorphic coronavirus particles from membranous material or other fringed bodies. The characteristic morphology of the core virion surrounded by a fringe of peplomers is not always apparent (Dea et al., 1981; Crouch and Acres, 1984).

An alternative method, the haemadsorption-elution-haemagglutination assay (HEHA) (Van Balken et al., 1978/79) relies on the virus' selective adsorption to and elution from rat red blood cells at different temperatures. The HEHA occasionally suffers from non-specific reactions due to the complex nature of faeces (Viscidi et al., 1984).

Additionally, several enzyme linked immunosorbent assays (ELISA) (Reynolds et al., 1984; Crouch et al., 1984) have been reported for detecting BCV but require highly specific antisera.

The detection of coronavirus antigen in cells of the infected gut using a fluorescent antibody technique has been described by Woode et al. (1978), but is dependent on tissue being removed very shortly after death and most antigen is present early in the course of disease. Coronavirus isolation in cell or organ cultures has been attempted but is also of limited value as many coronavirus strains do not grow readily in vitro (Bridger et al., 1978; Mebus et al., 1973).

In this paper an immunogold electron microscopy (IGEM) technique is described which was developed in an attempt to provide a rapid sensitive test that would be less susceptible to non-specific reactions. Immunogold labelling has already been shown to improve the detection of faecal viruses by increasing specificity and sensitivity (Kjeldsberg, 1985; 1986).

Materials and Methods

Virus preparation

Faeces from gnotobiotic calves experimentally and separately infected with BCV or rotavirus were used as positive and negative control samples respectively. Faecal samples were diluted 1 in 4 in phosphate buffer (PB), pH 7.2, mixed and ground with carborundum powder, centrifuged at 10000 × *g* for 3 min and the clarified supernatant fluid was examined for the presence of BCV.

Bovine coronavirus was also grown in vitro on fetal tracheal organ culture as described by Scott et al. (1976); and in human rectal tumour (HRT 18) cells as described by Laporte et al. (1979). Culture fluid was clarified by centrifugation at 10000 × *g* for 1 min and the supernate examined.

Antisera

Antiserum was produced in a gnotobiotic calf which had been infected orally with BCV at 4 days of age and given an intramuscular injection of BCV in Freund's complete adjuvant 4 wk later. Serum was collected 2 wk later and IgG extracted by affinity chromatography (Hudson and Hay, 1980). In addition, IgG from an anti-BCV serum produced in a gnotobiotic piglet was extracted by ion exchange chro-

matography (Hudson and Hay, 1980). The bovine and porcine sera had neutralising antibody titres of 1/1280 and 1/3840 respectively and were used at a dilution of 1/100 in all immunosorbent electron microscopy (ISEM) and IGEM tests.

Direct electron microscopy

Formvar carbon-coated 400 mesh copper EM grids were used throughout. A 3.5 μl drop of poly L-lysine was added to each grid and excess fluid was blotted with filter paper. A 3.5 μl drop of fluid to be examined was placed on each grid as appropriate and the excess removed with filter paper. The grids were negatively stained using phosphotungstic acid and ammonium molybdate (2:1).

A set pattern of examining EM grids was adhered to in order to standardise the counting of virus particles and facilitate comparison between different preparations. Virus particles were counted around the 4 edges of a grid square and across one diagonal. A total of ten grid squares from different areas of 2 duplicate grids was examined. The grids were coded and examined at a magnification of 20000 on a Siemens Elmiskop 1A electron microscope.

Immunosorbent electron microscopy

Copper EM grids were floated on a 10 μl drop of bovine anti-BCV IgG on a block of dental wax and incubated. The grids were then washed twice in PB and refloated on a 10 μl drop of antigen. After a second incubation the grids were negatively stained as previously described for DEM.

All incubations were performed in a moist chamber at 37°C for 1 h.

Immunogold labelling and the IGEM test.

A copper EM grid was coated with the porcine antibody by floating the grid on a drop of the pig anti-BCV IgG and was incubated for 1 h. The grid was washed twice with PB and reacted with a drop of antigen. After incubation for 1 h the grid was washed as described earlier and then floated on a drop of bovine anti-BCV IgG, reincubated for a further 30 min and washed as described previously. The grid was then placed on a drop of pig anti-bovine IgG which was conjugated to 5 nm colloidal gold. The conjugation was performed following the technique described by De Mey (1984). This conjugate was stored at 4°C and was diluted 1/5 in PB immediately before use. Any aggregates which may have formed on storage were removed by centrifugation of the diluted conjugate at 10000 × *g* for 2 min. After incubation with the gold conjugate for 1 h excess fluid on the grid was removed by blotting the side with filter paper and the grids negatively stained as described.

All incubations were performed in a moist chamber at 37°C. and a standard drop of 10 μl was used throughout.

During examination only virus particles with more than 20 gold particles attached to them were counted as positive to reduce the possibility of confusing light background staining with specific labelling.

Sucrose gradient fractionation of BCV

A volume of 60 ml of BCV grown in HRT18 cells was clarified by centrifugation at 3000 × *g* for 20 min to remove gross debris. Virus in the supernate was pelleted through a 10% (w/w) sucrose cushion by ultracentrifugation at 100 000 × *g* for 45 min at 4°C in a Beckman L5 Ultracentrifuge using an SW28 rotor. The pellet was resuspended in 0.3 ml TNE buffer (0.01M tris-hydrochloride (pH 7.5) – 0.1 M NaCl–1.0 mM EDTA), layered onto a 20–55% (w/w) sucrose gradient and centrifuged at 52 000 × *g* in an SW40 Ti rotor overnight at 4°C. The gradients were fractionated on an ISCO Density Gradient Fractionator and 0.5 ml fractions collected. Each fraction was examined by IGEM and the haemagglutination (HA) titre measured by the method described by Sato et al. (1977), using rat red blood cells.

Comparison of diagnostic tests

Fifteen bovine faecal samples were examined for BCV by DEM, ISEM, HEHA and IGEM. The HEHA test was performed as described by Van Balken et al. (1978/79). A further 94 faecal samples were compared by DEM and IGEM.

Results

Direct electron microscopy

It was often difficult to visualise intact virions and differentiate these from other fringed bodies by DEM. The low numbers of virus particles in addition to the presence of faecal debris compounded these difficulties and made unequivocal diagnosis of the presence of coronavirus difficult. Fig. 1A illustrates a coronavirus particle in a bovine faecal sample and highlights the problem of differentiating coronavirus-like particles.

Immunosorbent EM

The virus was concentrated approximately one thousand-fold using ISEM. The faecal background debris was markedly reduced but fringed bodies and virus-like particles were still detected in around 30% of samples, sometimes making interpretation difficult. The concentration effect of ISEM is illustrated in Fig. 1B.

Immunogold EM

Using IGEM BCV particles were both concentrated and specifically labelled. As illustrated by Fig. 1C, most of the gold particles were closely associated with the virus particles, with minimal gold background staining.

Fig. 1D illustrates a negative sample in which relatively low gold background staining was observed and debris or other particles which may have been mistaken for coronavirus were not labelled. Compared to DEM the background debris was also substantially reduced.

Control experiments utilised cultured and faecal BCV, and a faecal rotavirus preparation (Table 1). These control experiments showed that all the virus particles in the test (Table 1, column 1)., were specifically labelled. In the coating an-

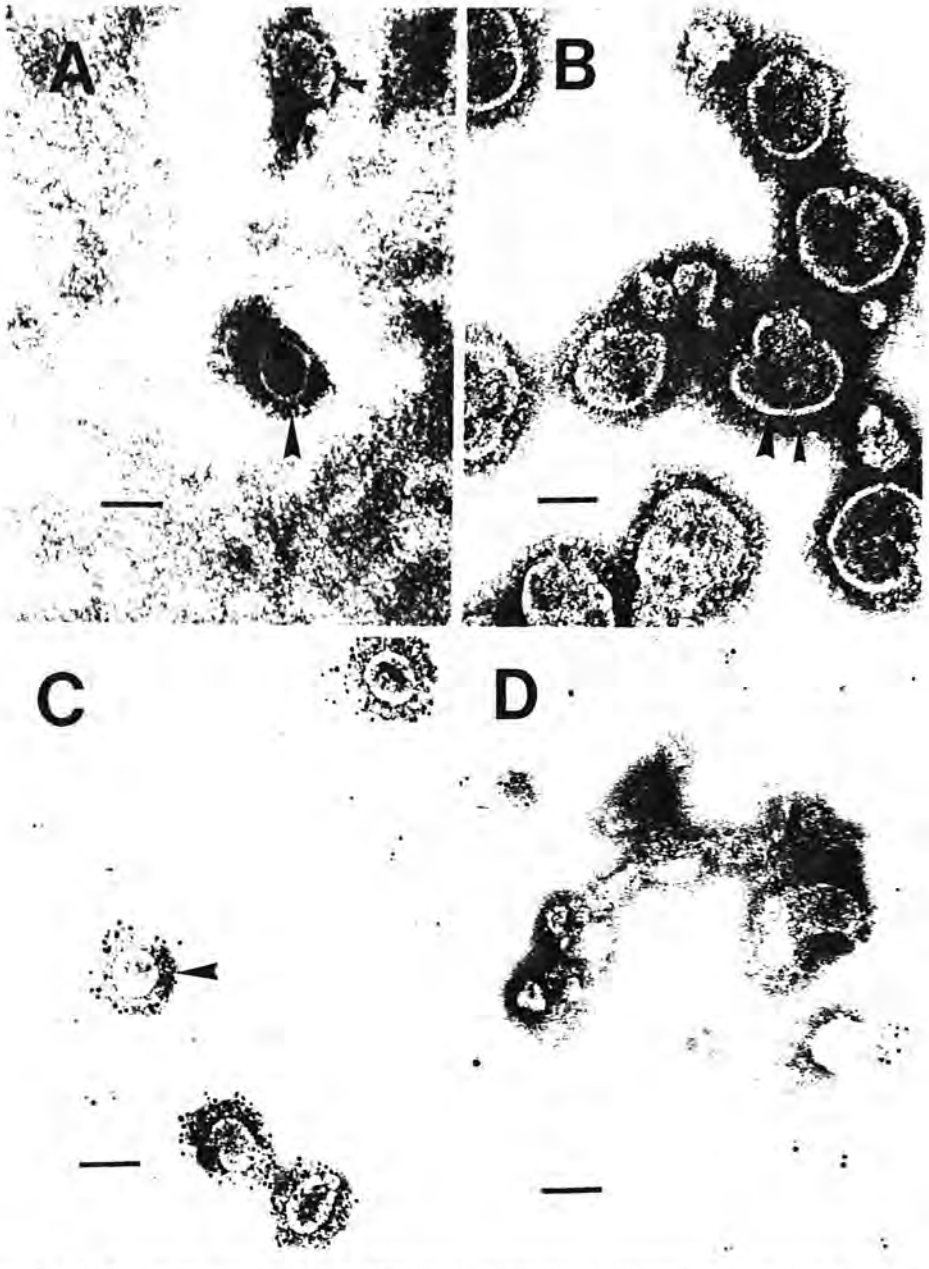

Fig. 1. (A) A particle considered to be BCV (large arrow) and a coronavirus-like particle (small arrow) observed by DEM. The potential for misdiagnosis is clear. (B) BCV particles detected by ISEM. The pleomorphic coronavirus particles (large arrow) are concentrated onto the antibody coated grid. In addition, many intact peplomers (small arrow) are obvious. (C) An IGEM preparation of a bovine faecal sample containing BCV. The 5 nm colloidal gold particles are mainly bound to the coronavirus particles (arrowed). (D) A bovine faecal sample, negative for BCV, as observed by IGEM. Fringed coronavirus-like particles are not labelled. Bars represent 100 nm.

TABLE 1

Number of coronavirus and coronavirus-like particles in 10 grid squares observed in IGEM and control preparations.

Sample	Technique			
	IGEM	IGEM performed without		
		1st Ab	2nd Ab	1st and 2nd Ab
Cultured BCV	3,556/0	0/1	0/2,943	nt
Faecal BCV	6,225/0	6/3	0/1,344	0/0
Faecal rotavirus	0/0	0/4	0/1	0/4

Ab : antibody. nt : not tested. −/− : no. of labelled particles / no. of unlabelled particles

tibody control, the results were similar to DEM examination, with no concentration of particles (column 2). Where the second antibody was omitted (column 3), results similar to ISEM were obtained, with concentration of the sample but no specific labelling. Finally by omitting both antisera (column 4), no concentration and no specific labelling were observed. These results demonstrate the specificity of the IGEM test.

Sucrose gradient

The number of labelled virus particles in sequential fractions of a sucrose gradient and the HA activity of each fraction are given in Fig. 2. The fractions containing the highest numbers of virus particles coincided with the peak of HA ac-

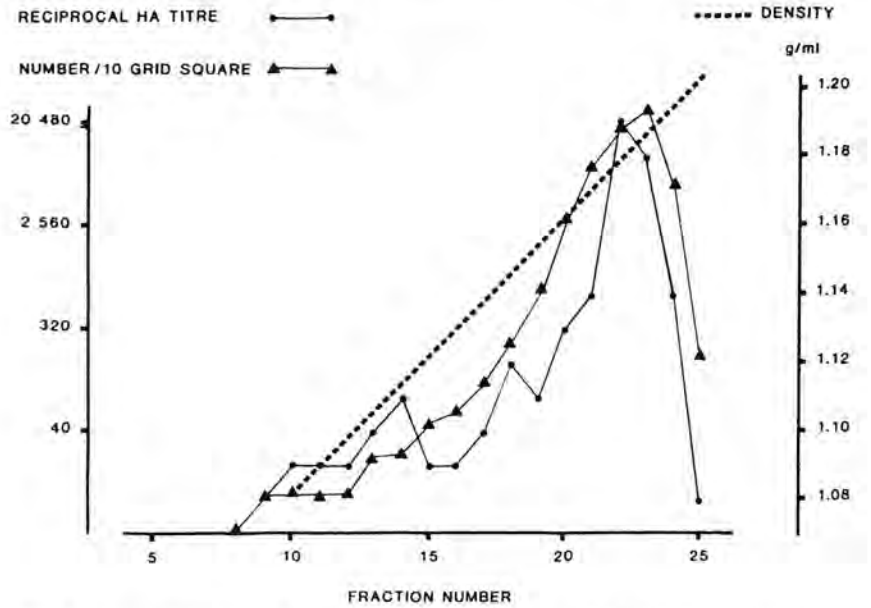

Fig. 2. HA and IGEM results on a 20–55% (w/w) sucrose gradient BCV preparation.

tivity. The density of this region was 1.18 g ml^{-1} which corresponds with that of BCV in sucrose (Siddell et al., 1983).

Comparison of diagnostic tests

Of 15 samples examined, coronavirus was detected in 2 samples by DEM, in 3 samples by ISEM, in 5 samples by HEHA and in 6 samples by IGEM (Table 2).

In the comparison of 94 faecal samples, 68 samples were found to be negative for BCV by both DEM and IGEM. Of the remaining 26 only 14 were positive when examined by DEM, whereas all were found to be positive in the IGEM test (Table 3).

Discussion

It has been noted that BCV peplomers are fragile and easily sheared from the outer envelope leaving a much less distinctive particle (Stair et al., 1972). Our results confirmed that when faecal samples containing BCV were examined by DEM coronavirus particles were usually observed to be widely spaced and the background heavily contaminated with debris. Equivocal results were compounded by the presence in faeces of other fringed particles which could be mistaken for coronavirus (Dea et al., 1981).

In this study a higher sensitivity and specificity were observed in ISEM prepa-

TABLE 2

Comparison of four tests for diagnosis of BCV in faeces.

Faecal sample	Detection of BCV by			
	DEM	ISEM	HEHA	IGEM
1	+	+	+	+
2	+	+	+	+
3	−	+	+	+
4	−	−	+	+
5	−	−	+	+
6	−	−	−	+
7–15	−	−	−	−
Total +	2	3	5	6

TABLE 3

Comparison between DEM and IGEM in the diagnosis of BCV in 94 faecal samples.

Results	No. of samples
DEM + IGEM +	14
DEM + IGEM −	0
DEM − IGEM +	12
DEM − IGEM −	68

rations. However around 30% of faecal samples still contained virus-like particles which could not be unequivocally determined to be BCV.

An attempt was made to further increase the sensitivity and specificity of BCV diagnosis by combining the concentrating effect of ISEM with the specific labelling of antibody conjugated to colloidal gold in an IGEM test. This was successfully achieved, with the IGEM allowing coronavirus-like particles to be diagnosed accurately as BCV or debris. The results of the coronavirus purification on a sucrose gradient gave further confirmation of specificity. The peplomers of coronavirus particles were rarely seen in IGEM preparations as the antibody coat seemed to obscure these projections. The main disadvantages of IGEM are the expense of reagents and equipment and the limited throughput of samples. IGEM should detect different BCV strains as isolates examined so far have been serologically similar (Dea et al., 1982).

The principle of this immunogold detection technique can be applied to other antigens which are not morphologically distinct and need to be labelled or localised. The technique therefore has widespread applications (Horisberger, 1981; Ducatelle et al., 1984) and has been used to label rotaviruses and adenoviruses (Kjeldsberg, 1985) in human faeces by an indirect labelling test where the viruses were directly adsorbed onto EM grids. Caliciviruses (Kjeldsberg, 1986) in human faeces have been labelled in suspension and the complexes adsorbed onto EM grids.

This novel technique should prove useful in the epidemiological study of disease associated with BCV infection, and as a standard against which to evaluate the development of other diagnostic techniques, such as the new dot-blot hybridization assay to detect viral RNA using a cDNA probe (Shockley et al., 1987).

Acknowledgements

The authors gratefully acknowledge Mr. A. McL. Dawson for IgG extraction; Mr. E.W. Gray and Miss L. McGee for expert advice and assistance with the electron microscope; Mr. A. Inglis for the photography; Mr. J.D. Menzies for producing the gold conjugate; Dr. Janice Bridger at the Institute for Animal Health, Compton Laboratory, for providing the porcine serum; and Dr. E. Davies at the PHLS, Bristol for supplying HRT18 cells.

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